HYPOLIPIDEMIC MODE OF ACTION OF 3-HYDROXY -3-METHYLGLUTARIC ACID

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

FACULTY OF SCIENCE

Aligarh Muslim University, Aligarh

BY

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DEPARTMENT OF CHEMISTRY

Aligarh Muslim University, Aligarh

1980

ABSTRACT

1. The dose-response effects of 3-hydroxy-3-methylglutaric acid (HMG) and nicotinic acid were studied on various tissue lipids in hyperlipidemic rats. Two doses of HMG (10 mg and 20 mg) and nicotinic acid (40 mg and 80 mg) per kg body weight per day, single and combined were simultaneously administered i.p. for 4 weeks. The lower doses reduced significantly serum triglycerides only, whereas, the higher doses of these compounds significantly decreased both cholesterol and triglycerides in serum, liver and aorta. The observed reduction on HMG administration seems to be dose-dependent. The combined lower doses of the two compounds significantly decreased all the lipid parameters in all these tissues. The reduction in tissue lipids was more marked by the administration of combined higher doses of the two compounds. However, the phospholipids remained unchanged by any dose used. The hypolipidemic effect by combined doses of HMG and nicotinic acid was found to be synergistic.

2. HMG effectively counteracted the olive oil-induced hyperlipidemia within 2-4 hr of its administration. The significant decrease in serum triglycerides, free fatty acids and phospholipids has been interpreted in terms of the inhibitory effect of HMG on VLDL production and intestinal absorption of fats.

3. Administration of HMG for 5 days effectively counteracted the diabetogenic action of alloxan in rats. The elevation of serum cholesterol, triglycerides, phospholipids, free fatty acids and blood glucose in diabetes were lowered by HMG treatment. Hepatic cholesterol of diabetic rats was suppressed. Liver, triglycerides and phospholipids were virtually unaltered. The possible mechanism of action of HMG in opposing the effects of diabetes is discussed.

4. HMG reversed the changes in different lipid fractions in serum and liver of carbon tetrachloride injected animals both in 48 and 168 hour study. Administration of HMG for a longer period resulted in a pronounced increase in the protective effect of HMG against carbon tetrachloride induced fatty liver. This action of HMG might be due to its inhibitory effect on depot fat mobilization and/or due to inhibition of VLDL production. The liver weight was significantly lowered in HMG-treated animals in both the studies indicating a beneficial effect of HMG.

5. Intramuscular administration of cobalt chloride resulted in a marked increase in different lipid levels in serum and liver. Treatment of rats with HMG effectively counteracted the enhanced lipemic response of cobalt chloride. All the lipid parameters decreased significantly in both the tissues. It was concluded that the hypolipidemic activity of HMG is caused by inhibition of synthesis of VLDL rather than the release of VLDL from liver.

6. Age-related alterations in the responsiveness of mature rats to HMG were evaluated in 2-3; 5-6; 9-10 and 15-20 months old rats. The concentration of cholesterol, triglycerides and phospholipids increased with age in serum and liver. Administration of HMG for 1 wk caused a decrease in these lipids in different age groups. The maximum effect was observed in serum triglycerides in all the groups. Insignificant differences in responsiveness between the age groups of approximately 3 and 9 month old rats were evident. Several mechanisms are probably involved in this phenomenon, including alteration in the compound distribution, metabolism, elimination and target organ sensitivity.

7. Oral administration of HMG for 1 wk to normocholesterolemic chickens significantly lowered serum cholesterol and triglyceride levels. The free fatty acids and phospholipids were insignificantly decreased. The lipid lowering action of HMG does not appear to be species-specific.

8. HMG significantly prevented the rise in serum cholesterol, triglycerides and phospholipids in Triton-induced hyperlipidemic chickens. An insignificant effect was found in free fatty acids. HMG effectively counteracted the hyperlipidemic response only when given along with Triton. The possibility of HMG exerting its hypolipidemic effect through the inhibition of lipoprotein synthesis appears more plausible.

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C E R T I F I C A T E

This is to certify that this thesis is the original work of MRS. RAHAT JAHAN KHAN, done under my supervision, and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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ACKNOWLEDGMENTS

I am extremely indebted to Professor Abdul Majid Siddiqi for his inspiring guidance, encouragement and direction throughout the period of this investigation.

I am thankful to Professor Wasiur Rehman, Head Department of Chemistry, A.M.U. Aligarh for providing necessary facilities.

It is with gratitude that I extend my thanks to Dr. S.Y.K. Yousufzai for his help and logical criticism during the course of this study.

I am extremely grateful to Drs. M. Saleemuddin, S.M. Hadi, H. Hague and A. Salahuddin for their help and interest in my work.

I sincerely acknowledge my husband, Dr. M.N. Khan for the immense co-operation and salutary help during the preparation of this thesis.

I owe much to my colleagues Drs. A.N.K. Yusufi, F.R. Khan, S. Siddiqi, M.T. Siddiqi, Z. Ahmad, A.A. Wani, Messrs. S. Umar, S. Ahmad, S.B.A. Zaidi, Nasir S. Ahmad, Drs. Nazma Sajid, B. Bano, R.Y. Rizvi, Ms. S. Malik, M. Alam and K. Varshney.

I express my thanks to Messrs. Iltija Khan, Bashir Ahmad and Noor Mohammad for their valuable technical assistance.

Thanks are also due to the Council of Scientific and Industrial Research (India) for financial support.

Rahat J. Khan

(RAHAT JAHAN KHAN)

CONTENTS

PREFACE

What causes heart attacks? A host of reasons have been cited by biological scientists in the past —— heredity, obesity, tension, smoking, lack of exercise, coffee drinking etc. Today biochemist's opinion is veering around to the view that the most important causative factor may be high blood cholesterol. Heart disease has been identified as number one killer in the United States, although it may be a more serious problem for under developed countries like India where industrialization is rapidly changing the life style. Retrospective and prospective studies have documented the close association between hyperlipidemia and the incidence of coronary heart disease (CHD). The implication of blood lipids as a contributing factor in the pathogenesis of atherosclerosis has led to a wide search for effective measures which control the concentration of cholesterol and triglycerides in blood and other tissues.

Numerous physiological and non-physiological lipid lowering agents have been investigated but due to side effects their fate in the successful chemotherapy of atherosclerosis and allied diseases is still debated. It is believed that an inhibitor of cholesterol biosynthesis, acting prior to HMG-CoA reductase step which is considered to be the main regulatory point in hepatic cholesterogenesis, cannot be

successfully used as a hypocholesterolemic drug because it would also affect other important metabolic reactions of the body. 3-Hydroxy-3-methylglutaric acid (HMG) formed in vivo by deacylation of HMG-CoA, has been shown to inhibit bacterial HMG-CoA reductase. In extensive studies in rats, rabbits and man, HMG has been found to possess potent hypolipidemic activity. The so far completed study show that compared to other hypolipidemic compounds, relatively small doses of HMG (20 mg/kg) cause maximum cholesterol and triglyceride decrease in serum and serum β-lipoproteins of mammalian system. It is believed that the mechanism of action of HMG in inhibiting the development of atherosclerosis might involve a shift of lipoprotein spectrum which may also partially be responsible for decreased cholesterol deposition in liver. However, in reducing serum cholesterol, an increased rate of excretion of cholesterol and/or its metabolite cannot possibly be excluded. Taking into account the potential hypolipidemic properties of HMG in rats and rabbits, and the effectiveness of this compound in the treatment of familial hypercholesterolemia, the present investigation has been directed to elucidate the possible mode of action of HMG. The following studies have been carried out.

(I) Dose-response effect of single and combined HMG and nicotinic acid treatment in hyperlipidemic rats.

(II) Effect of HMG on intestinal absorption of fat (olive oil-induced hypertriglyceridemia).

(III) Protective effect of HMG in alloxan-induced diabetes in rats.

(IV) Effect of HMG in preventing carbon tetrachloride-induced fatty liver in rats.

(V) Effect of HMG on cobalt chloride-induced hyperlipidemia in rats.

(VI) Age-related responsiveness of rats to hypolipidemic action of HMG.

(VII) Effect of HMG on serum lipids of normocholesterolemic chickens.

(VIII) Effect of HMG on serum lipids of chickens treated with Triton WR-1339.

C H A P T E R I

I N T R O D U C T I O N

GENERAL

Hyperlipidemia means too much lipid in the blood. At first sight this seems simple and straight forward, but in fact it is not. The situation is complicated because -

1. There are several different plasma lipids.
2. The plasma lipids do not occur in free form but are combined with proteins to form lipoproteins i.e., the condition can be better described as hyperlipoproteinemia (HLP).
3. "Too much" is very difficult to specify since we can not easily define normal values.

Most of the lipids in plasma are present as lipoproteins (Fredrickson et al., 1967). The levels of these lipoproteins are subject to dynamic changes. In hyperlipidemic conditions the concentration of one or more type of lipoproteins are elevated. The major lipoprotein families separated according to electrophoretic mobility or density, are inter-related within a complex metabolic system. The biosynthetic pathway of very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) is of particular interest in the study of hyperlipoproteinemia and hypolipoproteinemic drugs.

Retrospective and prospective studies have documented

a close association between hyperlipidemia and the incidence of coronary heart disease (CHD). Since only plasma cholesterol has been determined in most of these studies, it is not yet clear which type or types of hyperlipoproteinemia are most associated with CHD. From these studies and from those of Ghirardi et al.(1972) and Tzagournis, (1978), it is clear that not only plasma cholesterol but also fasting plasma triglycerides, with or without concomitant plasma cholesterol elevation, are closely associated with CHD. This indicates that elevation of the triglyceride rich VLDL is also involved in the association between CHD and HLP.

More than seventy years ago, the field of experimental atherosclerosis was opened by the studies of Ignatowski (1909). Since then, much efforts have been devoted to elucidating the mechanisms of genesis and the underlying causes of the atherosclerotic process by reproducing the disease in animals.

Atherosclerosis has been defined by the World Health Organization, as a "variable combination of changes of the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissues and calcium deposits associated with medial changes". Atherosclerosis is, therefore, a complex and multifaceted condition which may be collection of pathological

states with overlapping similarities of arterial abnormalities. Generally atherosclerosis either of aorta or of coronary arteries involves the formation of lipid deposits inside the walls of blood vessels. The developing atheroma has many of the features of a specific granulomatous lesion (Kaunitz, 1978). The early changes of the mesenchyma have been particularly well studied by Hauss (1973). The intima is invaded by smooth muscle cells (Koss and Glomset, 1973; Benditt, 1977). Gradually the characteristic plaque develops made up of the modified smooth muscle cells, collagen, debris from dying cells and varying amounts of lipids including cholesterol. The forerunner of atherosclerotic lesion can be recognized in the form of fatty streaks. It is believed that 90% of all genuine forms of angina pectoris and myocardial infarction are associated with sclerotic changes in the coronary artery (Schettler, 1961). The arteries afflicted with atherosclerosis exhibit thickening of the arterial wall intima, usually hypertrophy of the media with clearly demonstrable lipid deposits. Since all these factors lead to decrease in the size of arterial lumen, it results in the restriction of flow of blood at reasonable pressures. Thus the restricted supply of oxygen and nutrients to coronary muscle leads to diminished performance and finally heart failure.

A. EPIDIMEOLOGY OF ATHEROSCLEROSIS

It is now well recognized that a number of "risk factors" may be involved in the development of atherosclerotic heart disease. These factors include hypercholesterolemia (serum cholesterol, 260 mg/100 ml or above), hypertension (diastolic blood pressure of 95 mm or above), marked obesity, heavy cigarette smoking, diabetes mellitus, and a family history of vascular disease (Kannal et al., 1971; Lewis and Naito, 1978).

A number of undeniable facts have emerged which do show that cholesterol is somehow associated with degenerative disease.

(i) Atherosclerotic plaques contain large amounts of cholesterol and its esters;

(ii) Persons with sufficiently elevated serum cholesterol values have a higher risk of developing such atherosclerotic complications as heart attacks and strokes;

(iii) Disease associated with high serum cholesterol levels (e.g., nephrosis) are associated with pronounced atherosclerotic lesions and

(iv) Feeding of cholesterol to some mammalian species induces deposition of cholesterol in many tissues including arteries.

B. DIET AND ATHEROSCLEROSIS

A number of epidemiological and nutritional studies suggest a correlation between CHD and diet. Increase in CHD has been linked with increase in consumption of dietary components viz; cholesterol, animal protein and carbohydrates. Hyperlipidemia is produced by a variety of factors including increased absorption of dietary fat, altered transport of plasma lipids and changes in the composition of plasma lipids. Although the metabolic implications of each of these factors, as well as their possible relationship to atherogenesis differ, they have all formed the basis of a variety of systems for the listing of blood-lipid lowering agents. To some extent it is possible to produce experimental hyperlipidemia with predominant elevation of a given lipid class, although the lipid distribution is governed mainly by the composition of the type of lipoproteins that are elevated.

Atherosclerosis has been induced in rabbits (Kritchevsky, 1964; Constantinides, 1965), chickens (Dauber and Katz, 1942) monkeys (McGill et al., 1961), dogs (Steiner and

Kendall, 1946) and pigs (Downie et al., 1962) by cholesterol feeding. Kritchevsky et al. (1971) reported that rabbits fed a diet containing coconut or peanut oil with 2% cholesterol had the most extensive as well as the most frequent and severe lesions than that of corn oil. The corn-oil containing diet was less atherogenic than lard or butter oil, native or randomized, which have virtually no difference as regards their atherogenic potential (Kritchevsky and Tepper, 1977). It has been found that saturated fats along with cholesterol produced more severe atheroma than unsaturated fats (Kritchevsky and Tepper, 1967). Atherosclerosis can also be produced in rats within 5 days by intubating a mixture of cholesterol and vitamin D_2 in olive oil (Altman, 1973). There is a recent evidence for a more rapid metabolism of the abnormal cholesterol rich VLDL as compared to the control VLDL. The effect was found more pronounced in the lipoprotein fractions isolated from rabbits fed additional coconut or corn oil to their cholesterol enriched diets (Stange et al., 1975; 1977). This suggests significant implications on the atherogenecity of lipoproteins emphasizing their role in experimental atherosclerosis (Smith, 1978).

Recent evidences have shown that diets high in protein have a tendency to elevate the level of serum cholesterol (Carroll, 1978 a). It has been suggested that hypercholesterolemia has been related mainly to the use of casein as the source of protein (Carroll and Hamilton, 1975; Hamilton and Carroll, 1976). Other studies have provided evidence that a change from animal protein to plant protein in the diet is associated with a decrease in the level of serum cholesterol (Sirtori, 1977). It has been suggested that the level of plasma cholesterol in humans may be influenced by the kind of dietary protein as well as the amount in the diet (Carroll, 1978 b).

By feeding starch, sucrose, fructose, glucose or maltose containing diets, Naismith and Khan (1970 a, 1970 b) showed that animals eating simple sugars have higher triglyceride levels than those eating starches. This has been further confirmed by Nagarajan (1971) who showed greater incorporation of ^{14}C labelled sucrose, glucose, fructose into glyceride fraction in baboons. In experiments of this kind, carried out with several species including rats, spiny mice, pigs, and man, diets with sucrose produced an increase in the concentrations of

plasma cholesterol, triglyceride, and uric acid; a dimunition in glucose tolerance; an increased platelet adhesiveness; and a change in electrophoretic behaviour of platelets (Yudkin, 1972). In view of recent findings there is a stronger case for implicating sucrose in causing CHD than for implicating fat (Besset and Schroffner, 1968; Yudkin, 1978).

C. ABSORPTION AND METABOLISM OF FATS

For a number of years, investigations on the mechanism of fat absorption by the intestine have been primarily concerned with the analysis of the contents of the digestive tract and thoracic duct lymph following a fat meal. Dietary fat is absorbed after partial hydrolysis in small intestine. After reesterification to form triglycerides, it is packaged into spherical droplets (chylomicrons) in which the fat is coated with a monolayer of phospholipids, specific proteins and cholesterol. These droplets enter the lacteals of intestinal villi and are transported through the lymphatic system to the blood. Upon reaching the capillary bed of several extra-hepatic tissues, the chylomicrons are absorbed into the endothelial surface. As a result of the action of lipoprotein lipase the triglycerides are hydrolysed and liberated fatty acids are taken up locally into the tissues to be esterified and thus stored and oxidized for future energy needs. Alternatively, these fatty acids can recycle as free fatty acids in blood and enter tissues elsewhere.

Transport of exogenous triglycerides in chylomicrons is highly efficient so that chylomicronia ensues within few hours after each fatty meal. In states of genetically determined or acquired deficiency of lipoprotein lipase the capacity to transport triglycerides is quite limited and chylomicronia may persist from meal to meal (exogenous hyperlipidemia). In other more common forms of hyperlipidemia, not accompanied by over deficiency of lipoprotein lipase, the capacity to transport triglycerides in VLDL appears to be limited specifically (endogenous hyperlipidemia) so that concentration may be substantially increased when rates of production are in the normal range.

The level of VLDL and its constituent triglycerides can be affected by hormones that increase (norepinephrine, growth hormone) or decrease (insulin) the supply of fatty acids used for hepatic triglyceride synthesis. Insulin affects the level of triglyceride-rich lipoproteins by influencing the activity of lipoprotein lipase in extrahepatic tissues (Havel, 1965).Thus, the pathway of transport of triglycerides in blood plasma and its regulation provides a reasonably firm basis for evaluating the action of drugs that reduce triglyceride levels.

D. DIABETES AND ATHEROSCLEROSIS

It is well established that diabetes increases the risk of atherosclerosis in coronary, cerebral and peripheral arteries (Garcia et al., 1974; Keen et al; 1965). The coronary arteries and abdominal aorta of diabetics showed more atherosclerosis than non-diabetics regardless of age, sex, race and geographic location (Robertson and Strong, 1968). However, there is little evidence that within the diabetic population the frequency of large vessel complications is related to the degree of hyperglycemia, and treatment of hyperglycemia has not been shown to be protective against arterial disease (Stout, 1975). Hypertriglyceridemia is a common abnormality in diabetics (New et al., 1963) and is associated with atherosclerosis in diabetes (Santen et al., 1972). Elevated insulin levels found in many diabetics are related with other metabolic abnormalities such as obesity (Porte and Bagdate, 1970), hypertriglyceridemia (Bierman et al., 1970), and Uracemia (Bagdate, 1970). Chronic exposure to high concentrations of insulin results in the development of lipid filled lesions similar to those of early atherosclerosis (Renold et al., 1968; Stout, 1977). Insulin has the ability to promote

changes in the artery which, in the long term may progress to atherosclerosis (Stout, 1977).

Several groups of investigators have attempted to develop experimental models of diabetes. On the assumption that diabetes is a disease of absolute insulin deficiency, insulin secretion from the pancreas was ablated, either with the use of chemicals such as alloxan and streptozotocin, or by surgical removal of the pancreas. Alloxan or streptozotocin induced diabetic animals showed marked changes in lipid metabolism, especially an elevation in serum triglycerides (Meier et al., 1972) and a decrease in hepatic cholesterol synthesis (Corder and Kalkhoff, 1969). These compounds are selectively taken up by the insulin-producing β-cells of the pancreas and subsequently cause their destruction (Rerup, 1970). Previous work on the lipid levels of various tissues in alloxan-induced diabetes include decrease in the phospholipid of brain but not of liver (Karagezyan, 1968); increase in the free fatty acids and glycerides of the heart (Rizza et al., 1971); increased deposition of cholesterol in the retina (James and Alan, 1963); increase in cholesterol, phospholipid and triglyceride of liver (Malathy and Kurup, 1972) and increase in the lipid accumulation in the arterial wall(Stout, 1973; 1977). A decrease in lipoprotein lipase activity in liver

has been reported in diabetic rats (O'Conner et al., 1968; Malathy and Kurup, 1972). No change in lipoprotein lipase activity was observed by Shigeta et al., (1970), while Malathy and Kurup, (1972) found an increase in the enzyme activity in the heart. An increased myocardial enzyme activity was reported by Bondarenko (1970). Many hypolipidemic agents are known that inhibit the diabetogenic effect of alloxan and streptozotocin in rats (Benerji, 1947; Lazarow et al, 1950; Cayen, 1975; Duhault, 1976).

E. FATTY LIVERS

One experimental approach in the study of regulation of lipoprotein metabolism is through exploration of the mechanism of fatty liver production. Recknagel et al. (1960) suggested that a defect in excretion of lipids in the form of β-lipoproteins may be the underlying mechanism of fatty liver development. Inhibition in synthesis of the protein moiety of β-lipoproteins is the mechanism responsible for induction of fatty liver in response to several hepatotoxic drugs (Farber, 1966). An inverse relationship between hepatic triglyceride accumulation and serum lipoprotein concentration has been demonstrated in rats treated with puromycin (Robinson and Seakins, 1962), ethionine (Ugazio and Lombardi, 1965) and carbon tetrachloride (Rechnagel, 1967). However, synthesis of lipoproteins and their release from the liver appears to be a complex process (Oler and Lombardi, 1970). Feeding of orotic acid results in the development of fatty liver in rats (Standerfer and Handler, 1955; Handschumacher et al, 1960). This phenomenon unlike the development of fatty liver induced by choline deficient diet

does not seem to be accompanied by other serious pathological disturbances and is readily reversible. The feeding of orotic acid in diet selectively interferes with synthesis and/or release of VLDL without having any general effect on hepatic protein synthesis or on the release of other serum proteins synthesized in liver (Windmueller, 1964; Sidransky et al., 1963).

The adrenalectomy (Wool et al., 1954; Brody et al., 1961) and adrenergic blocking agents (Brody et al., 1961; Schotz and Page, 1959) have been found to prevent carbon tetrachloride and ethionine - induced fatty liver. It has been shown that administration of adenine or its nucleotide derivatives prevent the induction of fatty liver by ethionine, carbon tetrachloride, orotic acid (Handschumacher et al., 1960) and pectamycin, but failed to relieve inhibition of protein synthesis in vivo and the derangement of triglyceride transport from liver caused by the hepatotoxins (Glaser and Mager, 1972). According to Recknagel and Lombardi (1961) the formation of triglycerides by the liver is not interfered within the carbon tetrachloride poisoned animals, but the hepatic triglyceride secretory mechanism is inhibited. As a result, triglycerides accumulate in liver. It was first observed that carbon tetrachloride reached its peak

concentration in the liver of the rat within 1 to 2 hours after force feeding of the hepatotoxin (Recknagel and Litteria, 1960). The hepatic content of triglycerides was elevated 34% within 1 hour and 195% within 3 hours after carbon tetrachloride poisoning. This observation indicated that a serious derangement of hepatic lipid metabolism occurred coincident with the arrival of the toxic compound in the liver. A series of investigations (Recknagel and Anthony, 1959; Share and Recknagel, 1959) had previously led to the tentative conclusion that the hepatic parenchymal cell mitochondria were probably not the primary loci for the attack by carbon tetrachloride. The observation that carbon tetrachloride feeding results in pathological changes in the enzymatic properties of the microsome fractions at a time when the toxin is concentrated in the liver, and when rapid accumulation of triglycerides in the liver is taking place, suggested that the hitherto unknown hepatic mechanism, intimately associated with hepatic lipid metabolism and probably localized in the membraneous component of the endoplasmic reticulum, was most probably the key locus involved.

F. COBALT CHLORIDE - INDUCED HYPERLIPIDEMIA

Cobalt chloride has been known to produce hyperlipidemia in both man (Robey, et al., 1956) and animals (Munoz-Calvo et al., 1973; Kavacev et al., 1977). Several clinical reports of cobalt-induced goiter in human patients have made casual reference to the increased serum lipid values determined in the patients (Caren and Carbo, 1956; Carlson, 1963). Cobalt ion produces an endogenous hyperlipidemia which is virtually indistinguishable from "carbohydrate-induced lipemia" and is independent of dietary considerations (Eaton and Kipnis, 1969 a; 1969 b). The endogenous hyperlipidemia of both carbohydrate feeding and cobalt chloride treatment is initiated by insulin mediated stimulation of hepatic protein synthesis followed by release of the lipid carrying protein into the circulation (Eaton, 1972). The serum of rabbits was characterized as grossly lipemic with the predominant abnormality being an increase in VLDL transport protein and its triglyceride component. This led to the belief that cobalt chloride induced lipidemia may be secondary to changes in the metabolism of carrier protein. Daily intramuscular

injections of cobalt chloride to male rabbits for three days has been found to increase lipids, glucose and blood insulin levels in blood plasma and glucose-stimulated insulin secretion (Ohmichi, et al., 1974). The oral administration of cobalt chloride at the level of 10 mg/kg/day to male rabbits for 12-36 days increased the levels of cholesterol and β-lipoproteins in the blood serum; decreased the fat level in the liver and inhibited the lipolytic activity of the aorta wall and adipose tissues. A decrease in aorta wall lipoidosis was also observed (Lempert and Levina, 1974). When given orally at a dose of 3-6 mg/kg/day to rabbits with atherosclerosis induced by cholesterol, the effects on neutral fats and β-lipoproteins in serum were more pronounced (Boechko, 1972). Cobalt chloride, 25 mg/kg/day i.m. increased the blood plasma triglyceride level and turnover rate in rabbits. There was no change in lipoprotein lipase activity but there was an increase in liver triglycerides. It was suggested that the increase in blood plasma triglycerides caused by cobalt is a result of release of triglycerides from the liver (Morita et al.,1974). Plasma triglyceride concentrations in rats receiving cobalt chloride 40 mg/kg, s.c. for 10 days with an interruption of 9 days after the first injection was two-fold higher than in control rats, and the increased level of

triglycerides was maintained for at least one year. The increase in cholesterol and free fatty acids developed more slowly. Plasma triglyceride levels of anephric patients was increased during cobalt tretment, but unlike the level in rat plasma, the triglyceride level fell slowly toward the basal level after cessation of cobalt treatment (Taylor et al., 1977). Cobalt chloride also increased hepatic L-glycerophosphate and hepatic acyl CoA, and induced hyperglycemia and hypertriglyceridemia and increased plasma insulin levels. (Ohmichi, 1977).

G. TRITON-INDUCED HYPERLIPIDEMIA

The non-ionic detergent Triton WR-1339 injected intravenously, produces hyperlipidemia in rats (Nityanand and Kapoor, 1973), rabbits (Kellner et al., 1951 a), mice (Cornforth et al., 1951) and in monkeys (Kapoor and Nityamand, 1975). Yamada et al., (1966) reported a species difference in Triton induced hypercholesterolemia which was greater in rabbits than in rats, dogs and mice. Triton is believed to form a surface layer around lipoproteins and thereby protect them from enzymatic modification, specifically, in the case of VLDL, from hydrolysis by lipoprotein lipase. The marked hypertriglyceridemia has been attributed to failure of triglycerides to leave the plasma compartment (Lombardi and Recknagel, 1962). The lipids mobilized from tissue stores and bound in the plasma compartment by Triton are not metabolized, but may be deposited in the vessel wall. This process was demonstrated to occur in dogs and treatment with Triton was found to result in early atheromatosis (Scanu et al., 1961). In rabbits (Kellner et al., 1951 b) and rats (Still, 1962; Hess and Loustalot, 1963) the development of vascular lesions has been found to be

hindered by Triton in spite of the presence of hyperlipidemia.

Triton injected intravenously, has frequently been used to determine rates of triglycerides secretion into the plasma of experimental animals (Reaven et al., 1973). Illingworth et al, (1975) found that intravenous infusion of Triton WR-1339 (300 mg/kg body weight) increased plasma triglycerides almost linearly for 9-12 hours. Analysis of individual lipoproteins separated by ultracentrifugation showed that newly secreted triglycerides were present almost exclusively in the low density lipoprotein fraction. Illingworth (1975) also reported that in short-term experiments (3-6 hr) the detergent does not affect the rate of synthesis or secretion of these macromolecules into the circulation. Byers et al (1963) also failed to demonstrate any difference in the incorporation of ^{14}C-acetate into hepatic cholesterol from normal and Triton-treated rats 90 minutes after Triton-infusion; after 24 hr, however,cholesterol synthesis was increased in the Triton-treated animals. The Triton two-phase system has been used by Garattini et al. (1961) and Paoletti (1962) for testing potential hypolipidemic agents. Schurr et al., (1972) used Triton WR-1339 induced hyperlipidemia in rats as a ecreening model for various hypolipidemic agents..

H. TREATMENT WITH DRUGS

Though proof is still lacking, a considerable body of clinical and experimental evidence support the idea that correction or lowering of elevated plasma lipid levels may have a beneficial effect in the treatment of CHD (Levy and Fredrickson, 1970; Kannel et al., 1971). It is well known that effective inhibitors of cholesterol biosynthesis will lower not only cholesterol levels but also the levels of lipoproteins. Among the possible sites, interference with rate-limiting step in biosynthesis (HMG-CoA to mevalonic acid seems to be the most promising one (Gould, 1960; Migicovasky, 1962). The other possibilities, i.e., stimulation of cholesterol excretion and its degradation to bile acids, inhibition of absorption of dietary cholesterol, absorption or resorbtion of biliary cholesterol appear less feasible. A compound capable of lowering cholesterol may effect either one or more than one of the above processes.

Dietary regimes and drugs are the main methods used for reducing elevated plasma triglyceride or cholesterol levels in primary HLP. The former is often regarded as the

primary treatment of choice to be supplemented only if necessary by one or more drugs. Other types of treatments are small bowel resection and shunt operations, particularly effective in Type IIA HLP. In most cases, dietary treatment has to be supplemented by drugs to obtain substantial results.

Much has been learned about lipid transport physiology and lipid disorders in recent years. This in turn has shed light on the effect and mechanism of hypolipidemic agents. Table 1 gives a partial list of major hypolipidemic compounds in which the current interest continues.

1. Single Drug Treatment

Cholestyramine: Cholestyramine is a copolymer of divinyl-benzene with trimethylbenzylammonium groups, providing the exchange sites. It is hydrophillic but insoluble in water and remains unchanged in the gastro-intestinal tract. It acts by binding bile acids in the intestinal lumen and thereby preventing their resorbtion and promoting their excretion in the faeces. These sequestrating agents thus interfere with the enterohepatic circulation of the bile acids. As these are synthesized in liver from cholesterol, cholesterol catabolism is accelerated and as an effect plasma cholesterol concentration decreases. Cholestyramine was first used in patients with cholestasis in whom distressing pruritis prompted efforts to lower the content of bile acids in plasma (Carey, 1961).

Table 1. Hypolipidemic Compounds

I. Inhibitors of Cholesterol Absorption
Bile acid-binding resin (Cholestyramine*)
β-Sitosterol (Cytellin*)
Neomycin
Brain extract (Cerebrosides)
Aluminium oxide (Gelusil)
Androgenic steroids
Competing sterols (Dihydrocholesterol)
II. Inhibitors of Cholesterol Biosynthesis
Phenylbutyrates (Clofibrate, CPIB*)
Nicotinic acid (Heparinoid*)
3-Hydroxy-3-methylglutaric acid (HMG)
Triparanol (MER-29)
Aza sterols (7-Aza-5 β-cholestan-3 β-ol)
Ubiquinone
Hydroxylamines (Benzyloxy acetamide)
Cholestane-triol analogues
Benzmalacene
III. Enhancement of Cholesterol Excretion
Thyroactive substances (D-thyroxin*)
Salicylates
Linoleamides
Sulfaguanidine
Calcium carbonate
Nafoxidine HCl
Lipotropic agents (Choline)
IV. Unknown Mechanisms
Mucopolysaccharides (Mucin)
Phenothiazines
Vanadium
Biochanin A
Formononetin

* Clinically useful compound; commercial, common or specific names are given in parentheses.

Interest in the application of bile acid-binding resins in the treatment of hyperlipidemia quickly followed the demonstration that cholestyramine was effective in lowering of cholesterol and incidence of coronary atherosclerosis in cholesterol fed cockrel (Tennent et al., 1960) and atherogenic diet fed rats and rabbits (Beher et al., 1967). Plasma LDL levels decrease after treatment with cholestyramine while VLDL concentration may remain unchanged or increase (Levy et al., 1972). Treatment with 4 gm cholestryamine for two months decreased plasma cholesterol concentrations by 21% in Type II A and II B hypercholesterolemic patients (Carlson and Olsson, 1974). The effect of cholestyramine on serum lipids in patients with familial hyperbetalipoproteinemia has been evaluated in several short term studies (Jones and Dobrilovic, 1970; Levy et al., 1972). The average reduction of total plasma cholesterol in the 60 subjects of these studies, who received 12-24 gm of resin per day is 25%. Reductions of LDL cholesterol of 27% and 40% were observed on daily doses of 16 gm and 32 gm respectively.

Cholestipol, an insoluble tetraethylenepentamine and epichlorohydrin co-polymer is a new anionic bile sequestering resin which decreases resorbtion of bile acids and enhances their faecal excretion (Parsons, 1972). By increasing the

faecal excretion of bile acids, cholestipol brings about a compensatory increase in cholesterol production rate and an acceleration of its turnover. This results in a reduction of total cholesterol in tissue and plasma (Goodman et al.,1973; Miller et al., 1973). Recent studies have shown that cholestipol lowers plasma cholesterol levels in man and animals (Glueck et al., 1972; Fellin et al., 1975). The major drawback to the use of bile sequestering resin is the necessity of ingesting large doses of unpleasant tasting material. It also causes constipation, nausia, bloating and methiorism in patients. Nicotinic Acid and Derivatives: Nicotinic acid has been a consistently effective lipid lowering agent, when given in dose exceeding its requirement as a vitamin. Altschul (1956) found that the administration of nicotinic acid inhibited the development of both hypercholesterolemia and atheromatosis in cholesterol fed rabbits. It is now clear that plasma triglyceride levels are also markedly reduced by nicotinic acid (Carlson et al., 1968). In 1962, Carlson and Oro found that nicotinic acid caused a rapid and transient fall in the concentration of plasma free fatty acids, followed by a rebound. It was suggested that the reduction of free fatty acids may partly be the

cause of the reduction of plasma triglyceride and cholesterol levels. It was proposed that nicotinic acid inhibited mobilization of free fatty acids from adipose tissues. This reduced the uptake of free fatty acids in liver which resulted in decreased hepatic formation of VLDL. The consequences of these events would be to reduce the conversion of VLDL and eventually diminution of LDL level in plasma with the result that plasma cholesterol and phospholipids would be lowered. It appears that nicotinic acid effects the biosynthesis of cholesterol, probably prior to the cyclization of squalene (Goldsmith, 1962). There is also the possibility that nicotinic acid may act prior to the formation of mevalonic acid (Gamble and Wright, 1961). Barboriak and Meade (1971) showed reduction of usual alcohol induced enhancement of alimentary lipidemia in man by nicotinic acid administration. Similar results were also obtained in rats when they were given a corn oil-alcohol mixture by gastric intubation. Under these conditions less incorporation of ^{14}C-labelled dietary fat into plasma triglycerides was observed. Nicotinic acid, 3 gm daily, reduces plasma triglyceride and cholesterol levels by about 50-60% in Type III while it may reduce plasma triglycerides by 90% or more in Type V HLP (Carlson and Oro, 1973). Subcutaneous injection of nicotinic acid lowers the plasma VLDL and LDL concentrations,

but has no effect on plasma HDL in rhesus monkeys. It also diminishes the maximum incorporation of ^{14}C-labelled threonine into VLDL and LDL apoproteins, but has no effect on incorporation into albumin or HDL apoproteins (Amany et al., 1975). The Coronary Drug Project (1975) reported that nicotinic acid was the only hypolipidemic drug studied which significantly reduced the frequency of non-fatal recurrent myocardial infarction and cerebral vascular incidents. One advantage of nicotinic acid over clofibrate is that there is a dose-response relationship as far as the plasma lipid lowering effect is concerned. Benerji (1947) found that nicotinic acid, pyridinedicarboxylic acid and 2-Phenyl-quinolin-4-carboxylic acid are protective against alloxan diabetes. Lazarow et al., (1950), later on, confirmed that nicotinic acid in fact protected if given 60 minutes before alloxan. Nicotinamide but not nicotinic acid, was effective even if given immediately before alloxan, but only if the dose was increased about three times. The most common side and annoying effects of nicotinic acid therapy is intense cutaneous flushing and pruritis which generally develops within one to two hours of the dose. More troublesome effects may include gastrointestinal complaints, gastric distress, dispepsia, hyperuricemia and decreased glucose tolerance.

Many derivatives of nicotinic acid reduced the serum lipids without definite side effects. Complamin, a derivative of nicotinic acid, decreased blood cholesterol as well as other lipids (Blanco et al., 1963). Among nicotinic, nicotinuric and 3-pyridine acetic acid (100-250 mg/kg) given intraperitoneally to hyperlipidemic rats, nicotinuric acid was most effective (Brus, 1967). 2,2,6,6-tetrakis (nicotinonyloxymethyl) cyclohexanol (K-31) has been reported to suppress the elevation of serum cholesterol, phospholipid and triglyceride levels, but only cholesterol and phospholipids in liver (Aso et al., 1969). The hypochlolesterolemic action of K-31 may be due to inhibition of exogenous sterol absorption. Another derivative of nicotinic acid, pentaerythritol tetranicotinate (perycit) has been shown to possess hypolipidemic properties in high fat high cholesterol-fed rabbits (Brattsand and Lundholm, 1971). They showed that perycit reduced the rise in free and esterified cholesterol and triglycerides of serum. Perycit appeared generally to be somewhat more effective than nicotinic acid in reducing lipid infilterated area of aorta. Increase in dose of perycit (3 gm to 4.5 gm) caused a decrease in plasma lipid levels. In fat-fed rabbits, both drugs had a hypolipidemic and antiatherosclerotic effect when the plasma cholesterol value was below 1200 mg/100ml (Brattsand, 1975a). The antiatherosclerotic effect of niceritrol was partly attributed to its hypocholesterolemic effect, but it also seemed to have a more direct action on the vascular wall

(Brattsand et al., 1974; Brattsand, 1976). The reduction of plasma triglyceride and cholesterol concentrations can also be explained by decrease in VLDL triglyceride and cholesterol concentrations. The cholesterol concentration decreased in low density fraction but increased in high density fraction (Olsson et al., 1974). In a recent comparative study of nicotinic acid, niceritrol and β-pyridylcarbinol in guinea pigs fed hypercholesterolemic diet, 0.5-0.75% of β-pyridylcarbinol or niceritrol in the diet produced a long lasting and significant decrease of the plasma cholesterol by about 150 mg/100 ml, predominantly by reducing the raised plasma LDL, whereas nicotinic acid produced only a transient decrease. Niceritrol and β-pyridylcarbinol markedly reduced the lipid infilterated area of the abdominal aorta and the accumulation of free and esterified cholesterol in the abdominal aorta and coronary arteries, whereas nicotinic acid was less effective in the latter respect. Part of the antiatherosclerotic action of β-pyridylcarbinol and niceritrol was due to their hypocholesterolemic effect. A more direct action on vascular wall was probably also involved (Lundholm et al., 1978). Both perycit and (pyridine 2,5-dicarboxylic acid) di β-pyridylcarbinol ester (S-2042) in a dietary concentration of 0.5% decreased cholesterol. This reduction, as well as that induced by vitamins was confined to VLDL fraction. Only S-2042 slightly reduced the cholesterol accumulation in the aorta of atherosclerotic rabbits (Brattsand, 1975b).

Ethyl p-chlorophenoxyisobutyrate and Derivatives: The acceptance of this drug is based on its ability to reduce levels of both cholesterol and triglycerides (reflecting effects upon LDL and VLDL respectively) and upon its low order of toxicity. Ethyl p-chlorophenoxyisobutyrate (CPIB) is a branched fatty acid ester that can effect a wide range of metabolic activities of lipids and lipoproteins. These include reduced cholesterol synthesis (Thorp and Waring, 1962), interference with lipoprotein synthesis (Gould et al., 1967), inhibition of fatty acid synthesis (Maragoudakis, 1969), stimulation of lipoprotein lipase activity in adipose tissue (Tolman et al., 1970), stimulation of adenyl cyclase activity (Greene et al., 1970), and lowering of basal glycerol release from epididymal fat (Carlson et al., 1972). Investigations on lipemic rats (Segal et al., 1972) and lipemic man (Wolfe et al., 1973) have demonstrated improved removal of triglycerides from the plasma as a major response. However, in normal rats (Adams et al., 1971; Cenedella and Crouthamel, 1976) and man (Wolfe et al., 1973; Zakim and Herman 1969), not only enhanced removal of triglycerides is obvious, but also very little reduction in plasma triglyceride concentration is observed. In fact, reduction in hepatic triglyceride has been noted in normal rats without enhanced removal (Adams et al., 1971; Cenedella and Crouthamel, 1976). Evaluation of both production and removal of plasma triglycerides in normal and hyperlipemic rats, provide evidence that predominant lipid reducing

action of clofibrate is manifested in the hyperlipemic state, and predominently upon peripheral lipid disposal without altering triglyceride production (Simonelli and Eaton, 1978). CPIB inhibits cholesterol synthesis between acetate and mevalonate. It prevents the formation of fatty liver induced by orotic acid suggesting that the effect is mediated through a β-lipoprotein response (Westerfeld et al., 1972). Although earlier reports indicated that hypolipidemic action of CPIB is dependent upon the nature and type of diet (Zakim and Herman, 1969; Kokatnur and Malcom, 1970a; 1970b). It has been recently reported that the hypolipidemic action of CPIB is independent of the type of dietary fat in diet (Saito, 1975). Cayen (1975) found that CPIB lowered the elevated serum lipid levels in experimentally induced diabetic rats.

Various clofibrate analogues have been described as hypocholesterolemic and hypolipidemic agents in the treatment of atherosclerosis. A new derivative tetralylphenoxyisobutyrate (TPIA or SU-13437) was found effective in reducing serum triglyceride and cholesterol levels in Type III, IV and V hyperlipoproteinemia. Type II patients responded to a lesser degree (Hartman and Forster, 1969). According to Best and Duncan (1970) TPIA is more potent than clofibrate. However, a decrease of fibrinolytic activity was observed during the treatment with TPIA (Mannucci et al., 1971). Another analogue of CPIB, 1-methyl-4-piperidyl bis(p-chlorophenoxy) acetate (SaH 42-348) was found more effective in reducing serum

triglycerides and cholesterol than CPIB (Barkowitz, 1969; Timms et al., 1969). Halofenate, structurally related to clofibrate is a hypolipidemic and hypouricemic drug. In two short term clinical trials, it lowered the serum lipid levels of patients with hyperlipidemia (Sirtori et al., 1972). Newman et al. (1973) reported the hypolipidemic properties of certain acyclic and cyclic analogues of CPIB for Triton WR-1339 induced hyperlipidemic rats. The cyclic analogues include 1,4-benzodioxane and ethyl 6-chlorochroman-2-carboxymate. When CPIB and andostrone (Atromid S) are administered simultaneously, prolongation of the thrombin time, decrease of platelet adhesiveness, and increased fibrinolytic activity was observed. It has no influence on known components of the fibrinolytic system, when given alone (Sweet et al., 1965). CPIB has been associated with relatively overt side effects. It can occasionally cause nausia, diarrhoea and weight gain, but these usually disappeard quickly. Rare side effects include skin rash, weakness, giddiness and vomitting.

2. Drug Combinations

Until recently plasma lipid lowering drugs were used as single drug treatment, most often in combination with some dietary advice, but in recent years interesting reports on drug combinations have emerged. There are three reasons for combining two or more different plasma lipid lowering compounds.

(i) Treatment with one drug at its maximal dose does not always correct hyperlipoproteinemia. This is often the case in homozygous or pronounced heterozygous Type II A and in excessive Type IV.

(ii) The rate of occurrance of CHD is positively related to plasma triglyceride or cholesterol levels not only in hyperlipoproteinemia but also within the wide 'normal range'. Therefore, it seems logical to aim at maximal decrease of VLDL and LDL in order to prevent or delay CHD.

(iii) Some drugs show unwanted reciprocal lipoprotein effects. For example, treatment with clofibrate may increase LDL concentration in patients with low LDL Type IV pattern and treatment with bile acid sequestrants may result in increase in VLDL fractions in subjects with Type II A. Combination of drugs may eliminate these drawbacks.

Nicotinic Acid Derivatives plus Clofibrate: The effect of a combination of nicotinic acid derivative, pentaerythritol nicotinate, Perycit and clofibrate (Atromidin) was studied by Olsson et al. (1975). Since the standard dose of clofibrate is often not sufficient to normalize serun lipids, particularly in the treatment of serum cholesterol elevations, the effect of a combination with nicotinic acid was studied. It was found in this study that the combination of clofibrate and niceritrol had a pronounced effect on serum lipids and lipoprotein in 29 patients with Type II A and II B hyperlipoproteinemia. Niceritrol alone did not lower the serum triglyceride concentrations in Type II A with any dose. The combination of the two drugs reduced triglyceride concentrations by 30% and serum cholesterol by 22%. Combined treatment with 2 gm CPIB and 3 gm niceritrol resulted in a normal

lipoprotein pattern in 15 out of 17 subjects. No additional side effects were observed during combined treatment.

In an ongoing study by Carlson et al., (1977), five hundred and fifty eight consecutive survivors of myocardial infarction below 70 years (mean age 59 years) were randomly allocated into a control group and a chemotherapy group from December 1972 to April 1976. The chemotherapy group was prescribed clofibrate 1 gm twice daily, and nicotinic acid 1 gm three times daily. Serum cholesterol and triglycerides were lowered 15-20%, and 30% respectively in the chemotherapy group while only insignificant reductions were observed in the control group. Until December 1976, total mortality and mortality from ischemic heart disease had been the same in the two groups. The number of non-fatal myocardial infarction had been 38 in the control and 19 in the chemotherapy group, a statistically significant reduction.

In another study by Graham et al., (1977), forty-eight patients under 65 years were included in a double-blind study for comparison of the lipid lowering effect of clofibrate with that of β-pyridylcarbinol combined with clofibrate. Over four months there was no significant difference in the lipid lowering effect of either regime. A mean reduction of triglyceride of approximately 30% and of cholesterol of 18% was observed. Both the drugs caused significantly greater reductions than placebo.

Bile Acid Sequestrants plus Clofibrate: Howard and Hyams (1971) reported a good synergistic effect of the combination of PDX chloride (Secholex or DEAE-Sephadex) and clofibrate resulting in a total plasma cholesterol decrease of 34% after two weeks' treatment. In another study (Oliver, 1974) 16 gm cholestyramine (Questran) daily or 15 gm PDX chloride daily in combination with 2 gm clofibrate (Atromidin) for two months reduced the concentration of plasma cholesterol in Type II A hyperlipoproteinemic subjects. For cholestyramine plus clofibrate, the total plasma cholesterol decrease amounted 30% and the corresponding figure for PDX chloride was 24%. Comparing the effect of bile acid sequestrants alone, it seems that the addition of clofibrate has a marked effect in the PDX chloride treated group, thus compensating for the relatively poor effect of PDX alone.

Colestipol plus clofebrate: Recently Fellin et al. (1978) evaluated the effect of the association of cholestipol with clofibrate in the long-term treatment of familial hypercholesterolemia. Twenty subjects (12 Type II and 8 Type II B); previously treated with cholestipol for 16 months, were subjected to therapy with cholestipol (15gm/day) plus clofibrate (2 gm/day) for 15 months. In Type II A patients, the combination of these drugs enhanced the decrease in plasma cholesterol levels. In Type II B patients, on the other hand, the combination of clofibrate with cholestipol induced an increase in plasma cholesterol levels. A markedly significant decrease

in plasma triglyceride levels was observed in this group suggesting that, in Type II A, clofibrate enhances the resin's hypocholesterolemic effect. This is in agreement with the earlier findings (Goodman et al., 1973; Howard and Evans, 1974). In Type II B, on the other hand, the combination of these drugs did not seem to be indicated since a marked hypotriglyceridemic effect was accompanied by an increase in plasma cholesterol levels.

Nicotinic Acid plus Cholestyramine: Moutafis et al. (1971) showed that oral administration of cholestyramine with nicotinic acid decreased serum cholesterol in patients with familial hyperlipoproteinemia. Out of four subjects only one had a sustained reduction in serum cholesterol. They suggested that a combination of cholestyramine and nicotinic acid decreases the mass of exchangeable cholesterol in the body by increasing cholesterol removal and inhibiting the compensatory increase in hepatic cholesterol synthesis, that would otherwise occur in response to increased removal. The reports of another study indicate that the combination of nicotinic acid with a bile acid-binding resin may be a more effective approach to these subjects. In the study, five of six such patients had a sustained reduction in serum cholesterol of 33% below the level achieved with resin alone (Levy et al., 1972).

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Animals

Unless mentioned otherwise, adult male albino rats used in the present study were purchased from the stock colony of a local animal supplier. White Leghorn chickens were purchased from Vijay Poultry Farm, Aligarh (India).

2. Experimental Manipulations

The animals were conditioned for 2-3 weeks on basal diet prior to their transfer on experimental diet. For producing hyperlipidemic conditions, the animals were kept either on hypercholesterolemic diet or treated as described below. The diet and water were given ad libitum. The animals were always randomly divided into groups as indicated in Chapter III.

Basal Diet: Hind Lever Rat Feed and Hind Lever Poultry Feed was obtained from Hindustan Lever Ltd., India.

Cholesterolemic Diet: The following ingredients were mixed as w/w percentage (Fujiwara et al., 1972): casein 15; sucrose 67.1; hydrogenated vegetable oil 10; salt mixture[a] 4; cellulose 2; vitamin mixture[b] 0.5; chloride 0.2; cholesterol 1, and cholic acid 0.2.

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[a]The salt mixture contained (percent in the mixture): NaCl 4.6; $Na_2HPO_4.H_2O$ 9.3; K_2HPO_4 25.6; $CaH(PO_4)_2.H_2O$ 14.5; $Fe(C_6H_5O_7)5H_2O$ 3.2; $Ca(C_3H_5O_3)5H_2O$ 34.9; $MgSO_4$ 7.0; KI 0.9.

[b]One hundred gm of the vitamin mixture contained (in mg): riboflavin 150; thiamin 100; nicotinic acid 1000; pyridoxine 100; cyanocobalamine 1; pantothenic acid 500; folic acid 50; ascorbic acid 3750; vitamin E 100; Vitamin A 250,000 I.U.; Vitamin D_2 20,000 I.U. and sucrose to 100 gm

Intubation of Olive oil: To induce hypertriglyceridemia, the animals were loaded orally with olive oil at the dose of 20 ml/kg, (Duhault et al., 1976) 2 hour after drug administration.

Alloxan treatment: Diabetes was induced in rats by a single subcutaneous injection of 180 mg alloxan per Kg body weight. The rats were fasted for 48 hr before alloxan administration. Insulin injection i.p. 0.5 unit/130 gm body weight, was adjusted daily for glucosuria $^{++}$ or $^{+++}$ (test tape) and stopped 18 hr before sacrifice (Malathy and Kurup, 1972).

Carbon tetrachloride treatment: The animals kept on basal diet were given intramuscular injections of carbon-tetrachloride at the dose of 3 ml/kg per day (1:1v/v) in mineral oil. In 48 hr study, the injections were given at intervals of 0, 6, 24 and 30 hours. They received an injection every day in 168 hr study (Vaishwanar et al., 1972).

Cobalt Chloride treatment: To produce hyperlipidemic conditions, the animals were given intramuscular injections of cobalt chloride at a dose level of 25 mg/kg/day for seven days (Morita et al., 1974).

Triton Treatment: Chickens conditioned on basal diet received i.p. injection of 300 mg Triton WR-1339/kg body weight dissolved in 0.15 M sodium chloride (Schurr et al., 1972).

3. Sacrifice

To avoid possible differences due to diurnal rythm, all the animals, except in carbon tetrachloride and olive oil treatment, were sacrificed around 10.00 A.M. The animals fasted overnight were anesthetized with ether, blood withdrawn by cardiac puncture (allowed to coagulate before serum was separated by centrifugation), excised, tissue(s) removed, quickly washed, blotted, weighed and suspended in a mixture of chloroform-methenol (2:1, v/v) for lipid extraction as described later.

4. Chemicals

Special reagents obtained from commercial sources and used without further purifications included: 3-Hydroxy-3-methylglutaric acid (Schwarz/Mann, U.S.A.); Nicotinic acid (Nutritional Biochemicals, U.S.A.); 1-amino-2-hydroxynaphthalene-4-sulphonic acid (sodium salt); cholic acid and choline chloride (E. Merck, Germany); trichloroacetic acid and perchloric acid 70% (Riedel, Germany); cholesterol (J.T. Baker Chemical Co., U.S.A.); hydrogenated vegetable oil (Trade name 'DALDA' of Hindustan Lever Ltd., India); olive oil (Bertolli, Italy); Zeocarb-225 (Ion-Exchange Ltd., India); Triton WR-1339 (gift from Upjohn Co. Ltd., U.S.A.); Alloxan and cobalt chloride (B.D.H., India), cycloheximide (Sigma Chemical Co., U.S.A.). Rest of the chemicals and solvents were of reagent grade, glass distilled water was used throughout the study.

B. CHEMICAL METHODS

1. Extraction of Tissue Lipids:

Tissues were extracted for total lipids by the method of Folch et al. (1957). A known weight (0.3 - 1 gm) of tissue randomly selected, and cut from the tissue was grinded using pestle and mortar with 20 fold (w/v) chloroform-methanol (2:1, v/v) mixture. The homogenate was filtered through Whatman No. 1 filter paper into a glass stoppered tube. A suitable aliquot (6-10 ml) of this tissue extract was transferred to another glass stoppered tube. This crude extract was mixed thoroughly with 2 ml of 0.9% sodium chloride, and was allowed to stand for 5-6 hr till two phases without interfacial fluff separated. The upper aqueous phase was removed as much as possible by a syringe. The lower phase was gently washed 3 times with known amount of the salt solution by slowly adding it along the side of the tube by a swirling action of the pipette. This was done to avoid disturbance of the lower phase. Finally, the lower phase, without disturbing the remaining rising fluid, was made into one phase by addition of methanol (10-15 drops). The resulting solution was diluted to 10 ml in a standard flask by addition of chloroform-methanol mixture (2:1, v/v). This solution was then used for various lipid estimations.

2. Quantitative Determination of Total Cholesterol

The method used was essentially that of Zlatkis et al. (1953). A suitable volume of serum of lipid extract (0.05 to

0.5 ml) was added to 3 ml glacial acetic acid and mixed thoroughly. Two ml of ferric chloride reagent (freshly prepared by diluting 1 ml of 10% w/v, ferric chloride in glacial acetic acid to 100 ml with concentrated sulphuric acid) was carefully added from the side to allow the formation of a brown ring. The tubes were shaken thoroughly, cooled and colour density read in a Baush and Lomb 'Spectronic 20' spectrophotometer at 560 nm against a reagent blank. A cholesterol solution of known strength prepared in glacial acetic acid was used to prepare a standard curve. Under described experimental conditions, an optical density of 1.00 was equivalent to 232 µg cholesterol.

3. Quantitative Determination of Triglycerides

Total triglycerides were determined by the method of Van Handel and Zilversmit (1957). Four gm of zeocarb-225 placed in a glass stoppered 100 ml conical flask and was moistened with 2 ml chloroform. After adding a suitable aliquot (0.03-1 ml) or serum of lipid extract of tissue, 10-15 ml chloroform was added. The contents allowed to stand for 1 hour at room temperature (intermittent shaking was done), were filtered through a Whatman No. 1 filter paper, 2-3 ml aliquot of filtrate was transferred to three glass stoppered tubes and solvent evaporated. To two tubes 0.5 ml of 0.4% alcoholic potassium hydroxide (w/v) was added, but only 0.5 ml of alcohol was added to the third tubes (unsaponified sample). All the tubes were kept at 60-70^{o} for

15 minutes. 0.5 ml of 0.2 M sulphuric acid was added to each tube, which was placed in a gently boiling water-bath for 15 minutes to remove alcohol. After cooling the triglyceride content was determined by periodate oxidation. 0.1 ml of 0.05 M sodium periodate solution was added to the tubes and oxidation was stopped exactly after 10 minutes by the addition of 0.1 ml of 0.5 M sodium arsenite. A yellow colour of iodine appeared which disappeared within few minutes. Nine ml of 0.24% chromotropic acid reagent (in sulphuric acid and water 2:1, v/v) was added and heated for exactly 30 minutes in boiling water bath. The colour was read in Baush and Lomb 'Spectronic 20' spectrophotometer at 570 nm. The quantity of triglyceride is represented in terms of weight of corn oil which has been used as standard for triglyceride estimation. One optical density unit was equal to 188 µg corn oil equivalent triglycerides.

4. Quantitative Determination of Total Phospholipids

Total phospholipids were determined by the method of Bartlett (1959) as modified by Marinetti (1962). For direct determination from serum, 0.01-0.1 ml sample was delivered into centrifuge tubes containing 0.5 ml of water. Three ml of freshly prepared 10% (w/v) trichloroacetic acid solution was added. The tubes were allowed to stand for few minutes and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was decanted and tubes inverted on filter paper until practically all the supernatant was removed. For total

phospholipid determination from tissue lipid extract, a suitable aliquot (0.05-0.1 ml) was taken into a tube and evaporated to dryness. The contents of tubes were digested on an electric digestion unit with 1 ml perchloric acid (70%) for 15-20 minutes (2-3 glass beads of boiling chips were added in each tube to avoid bumping). On cooling, 7 ml distilled water was added followed by 1.5 ml of 2.5% (w/v) ammonium molybdate. The tubes were shaken thoroughly and 0.2 ml of 0.25% (w/v) 1-amino-2-hydroxynaphthalene-4-sulphonic acid was added. The tubes were heated in boiling water-bath for exactly 7 minutes, cooled and colour read after 20 minutes in Baush and Lomb 'Spectronic 20' spectrophotometer at 830 nm against a reagent blank. For a calibration curve monopotassium dihydrogen phosphate was used as standard. It was found that an optical density of 1.00 was equivalent to 10 µg phosphorus. The phospholipid values were obtained after multiplying the phospholipid phosphorus by a factor of 25.

5. Quantitative Determination of Blood Glucose

Serum glucose was determined by the method of Nelson (1944). 0.1 ml of serum was delivered into centrifuge tube containing 1.5 ml of water. 0.2 ml of 0.3 N barium hydroxide solution was added to the mixture followed by 0.2 ml of 5% solution of $ZnSO_4.7H_2O$, with thorough shaking. The tubes were allowed to stand for several minutes and then centrifuged at 3000 r.p.m. for 15 min. One ml of supernatent was then pipetted into a test tube graduated at 25 ml for further

analysis. One ml of alkaline copper reagent prepared on the same day by mixing 25 parts of copper reagent A*to 1 part of B** was added. The solutions were mixed and heated in a boiling water bath for 20 minutes. After cooling, 1 ml of arsenomolybdate reagent (containing 25 gm of ammonium molybdate in 450 ml water, 21 ml concentrated H_2SO_4 and 3 gms Na_2HASO_4 dissolved in 25 ml of water) was added. The reaction mixture was allowed to stand for a few minutes until the effervescene ceased. The samples were mixed thoroughly and diluted to 25 ml upto the mark. A stable blue colour quickly appeared which was read in Bausch and Lomb 'Spectronic 20' spectrophotometer at 600 nm against a reagent blank. A glucose solution of known strength was used to prepare a standard curve. Under described experimental conditions optical density of 1.00 was equivalent to 233 µg glucose.

6. Quantitative Determination of Free Fatty Acids

The free fatty acids in serum were estimated by the method of Koichi and Michio (1965). To a centrifuge tube containing 3 ml chloroform, was added 0.2 to 0.3 ml serum. The solution was shaken thouroughly and then centrifuged at 3,000 rpm for 10 minutes. The upper layer containing the protein precipitate was removed with the help of a syringe. 1.5 ml of chloroform layer was then pippeted into a glass

*Reagent A:
25 gm Na_2CO_3 + 25 gm Sodium potassium tartarate + 20 mg $NaHCO_3$ + 200 gm Na_2SO_4 (Anhydrous) in 800 ml of water and diluting to 1000 ml.

**Reagent B:
15% $CuSO_4.5H_2O$ containing 1 to 2 drops of H_2SO_4.

stoppered tube containing 4.5 ml chloroform. To this was added 3 ml of copper-triethenolamine solution (prepared by mixing 1 M triethenolamine, 1 N acetic acid and 6.45% $Cu(NO_3)_2 \cdot 3H_2O$ in the ratio of 9:1:10 v/v). The tubes were shaken mechanically for 10 min. After a settling period of 30 min. or more the copper-triethenolamine solution was aspirated with a syringe. To 4 ml of the chloroform layer, was added 0.4 ml sodium diethyldithio carbamate (0.1% in n-butanol w/v). The yellowish brown colour developed immediately was measured in Bausch and Lomb 'Spectronic 20' spectrophotometer at 440 nm against a reagent blank. Palmitic acid solution of known strength was used to prepare a standard curve. One optical density unit was found to be equivalent to 134.4 µg palmitic acid.

7. Treatment of Data

Unless mentioned otherwise, per cent reduction in tissue lipids was calculated in the following manner:

$$\text{Per cent Reduction} = \frac{\text{Value of control group} - \text{Value of HMG treated group}}{\text{Value of control group}} \times 100$$

All determinations were done in atleast duplicates. Statistical significance was calculated by Student's t-test.

C H A P T E R III

R E S U L T S

RESULTS

1. Dose Response Effect of Single and Combined HMG and Nicotinic Acid treatment on Various Tissue Lipids in Hyperlipidemic Rats

Seven groups, each of five rats, weighing about 100-150 gm were fed a high cholesterol diet as described in chapter II. Along with the diet the animals received each day i.p. HMG at the concentration of 10 and 20 mg/kg body weight in 1 ml saline. Two doses of nicotinic acid, 40 and 80 mg/kg in 1 ml saline were administered i.p. to two other groups. The rest two groups received a combination of 10 mg HMG plus 40 mg nicotinic acid, and 20 mg HMG plus 80 mg nicotinic acid/kg in 1 ml saline. The hyperlipidemic control group received i.p. 1 ml saline only. After two weeks of treatment, the animals were sacrificed. Various lipid parameters were determined in serum, liver and aorta (Table 2).

HMG Dose-Response Study:

As evident from Table 2, i.p. administration of HMG at a dose of 10 mg/kg significantly decreased serum triglycerides to the extent of 28%. The triglyceride levels in liver and aorta were, however almost not affected. An insignificant reduction was observed in cholesterol in serum, liver and aorta. HMG treatment at a dose of 20 mg/kg significantly lowered cholesterol to the extent of 40%, 18%

Table 2. Dose-Response Effect of Single and Combined HMG and Nicotinic Acid Treatment on Various Tissue Lipids in Hyperlipidemic Rats

Tissue Lipids	Hyperlipidemic control group	Treated Groups: 10 mg HMG per kg	20 mg HMG per kg	40 mg Nicotinic acid per kg	80 mg Nicotinic acid per kg	10 mg HMG + 40 mg Nicotinic acid per kg	20 mg HMG + 80 mg Nicotinic acid per kg
Serum (mg/100 ml)							
Cholesterol	663±70[a]	532±84 (20)[b]	395±12[d] (40)	528±33[h] (20)	405±60[d] (39)	332±24[d] (50)	246±48[d] (63)
Triglycerides	252±32	182±25[h] (28)	128±10[d] (49)	146±24[e] (42)	132±14[e] (48)	98±8[d] (61)	80±9[c] (69)
Cholesterol: Phospholipid ratio	1.31	1.04	0.79	1.04	0.83	0.69	0.51
Liver (mg/gm)							
Cholesterol	52±4	48±4[h] (8)	43±3[f] (18)	49±3[h] (6)	44±2[f] (15)	39±2[d] (27)	34±2[d] (35)
Triglycerides	10.8±1	10.7±1.5 (1.3)	8.2±0.9[g] (24)	10.4±1[h] (4)	8.6±0.6[g] (20)	9.4±1[h] (13)	7.6±0.66[f] (29)
Cholesterol: Phospholipid ratio	1.73	1.78	1.65	1.74	1.47	1.63	1.43
Aorta (mg/gm)							
Cholesterol	8.5±0.8	7.7±1.2[h] (9)	5.5±0.6[g] (35)	7.1±0.7[h] (17)	5.2±0.6[e] (39)	6.2±0.6[f] (28)	4.8±0.8[e] (44)
Triglycerides	111±3	111±13 -	85±3[c] (22)	109±6[h] (1.2)	86±4[d] (22)	93±2[d] (16)	78±3[c] (29)
Cholesterol: Phospholipid ratio	0.11	0.16	0.07	0.09	0.07	0.09	0.01

[a] Mean ± Standard Error expressed for five rats

[b] Values in parentheses indicate per cent reduction with respect to control group

[c] Significantly different from control group $p < 0.0001$; [d] $p < 0.001$; [e] $p < 0.01$; [f] $p < 0.05$; [g] $p < 0.02$; [h] not significant

and 35% and triglycerides to the extent of 49%, 24% and 22% respectively in serum, liver and aorta. The effect of HMG on cholesterol and tryglyceride levels were dose-related.

Nicotinic Acid Dose-Response Study:

We have confirmed the earlier findings of Olsson et al. (1975) that nicotinic acid has a dose-response effect on both serum cholesterol and triglycerides. Nicotinic acid at a dose of 40 mg/kg significantly reduced cholesterol and triglyceride levels in serum to the extent of 20% and 42% respectively, whereas, these lipid parameters in liver and aorta were insignificantly decreased. The higher dose of nicotinic acid i.e. 80 mg/kg significantly lowered cholesterol in serum, liver and aorta to the extent of 39%, 15% and 39% and triglycerides to the extent of 48%, 20% and 22% respectively.

Combined Effect of HMG and Nicotinic Acid:

Intraperitoneal treatment of a combination of 10 mg HMG and 40 mg nicotinic acid/kg resulted in a significant lowering of cholesterol in serum, liver and aorta to the extent of 50%, 27% and 28%, and triglycerides to the extent of 61%, 13% and 16% respectively. The combined higher dose of 20 mg HMG and 80 mg nicotinic acid significantly reduced cholesterol in these tissues to the extent of 63%, 35% and 44%, and triglycerides to the extent of 69%, 29% and 29%, respectively. The tissue phospholipids were, however, neither affected by single doses nor by any combined dose used. During the combined treatment, the effect on all lipid

Table 3. Effect of Single and Combined Doses of HMG and Nicotinic Acid on Wet Weight of Liver in Hyperlipidemic Rats

	Hyperlipidemic control group	10 mg HMG per kg	20 mg HMG per kg	40 mg Nicotinic acid per kg	80 mg Nicotinic acid per kg	10 mg HMG + 40 mg Nicotinic acid per kg	20 mg HMG + 80 mg Nicotinic acid per kg
Wet Weight of liver	5.68 ± 0.2[a]	4.56 ± 0.3	4.36 ± 0.4	5.52 ± 0.4	4.20 ± 0.6	4.52 ± 0.4	4.50 ' 0.3
		$p < 0.02$	$p < 0.01$	N.S.[b]	N.S.	$p < 0.02$	$p. < 0.01$

[a]Mean ± Standard Error expressed in grams.

[b]N.S. = Not significant

parameters in these tissues were more efficient than either compound in these doses. The effect on cholesterol level was more marked than with either compound alone. Similar results were obtained for triglyceride levels in these tissues.

On administration of 10 or 20 mg HMG in rats (Table 3), a significant decrease in average wet weight of liver was observed. There was no change in liver weight by the administration of either dose of nicotinic acid. Administration of combined lower or combined higher doses of the two compounds caused a significant reduction of the same magnitude as with 10 or 20 mg HMG/kg. There was no marked difference in dietary intake among control group and treated groups. In all groups the animals remained active throughout the experimental period.

Effect of HMG on Olive Oil - Induced Hypertriglyceridemia in Rats

Four groups of rats weighing 120-150 gm were fed basal diet and water ad libitum. They were fasted overnight prior to the start of experiment. The experiment was performed in two sets. HMG at the concentration of 50 mg/kg body weight in 1 ml saline was orally administered to the experimental animals in both the sets. Then after two hours, all the animals were loaded with olive oil, 20 ml/kg body weight by gastric intubation. Two hours later, the rats in the first set of two groups of 10 animals were sacrificed. The animals

Table 4. Effect of HMG on Olive oil-Induced Hypertriglyceridemia in Rats

Details	Triglyceride	Cholesterol	Phospholipid	Free Fatty Acids
Basal group	113.5 ± 7.2[a]	63.3 ± 4.8	81.6 ± 6.3	30.0 ± 1.7
First Set				
control group	266.6 ± 13.0	80.4 ± 2.6	129.4 ± 4.5	70.6 ± 2.2
HMG-treated group	217.3 ± 8.4	73.2 ± 5.0	96.2 ± 8.0	56.6 ± 3.4
	(19)[b]	(9)	(26)	(20)
p-value	$p < 0.01$[c]	N.S.	$p < 0.02$	$p < 0.001$
Second Set				
control group	245.0 ± 14.5	83.0 ± 3.7	124.0 ± 2.7	66.7 ± 1.2
HMG-treated group	170.4 ± 5.0	70.0 ± 2.0	92.8 ± 8.6	50.9 ± 1.3
	(31)	(16)	(25)	(24)
p-value	$p < 0.0001$	$p < 0.02$	$p < 0.01$	$p < 0.0001$

[a]Mean ± Standard error expressed for five rats

[b]Values in parentheses indicate per cent reduction with respect to control group

[c]N.S. = Not significant

of the second set were sacrificed six hour after HMG administration. The respective control groups were in two separate sets. They received an equal volume of saline in addition to olive oil for the same period. Five normal rats of similar body weight in basal group were also sacrificed at the same time for comparison of lipid values. Triglyceride, phospholipid, cholesterol and free fatty acid levels were estimated in serum.

The data of this experiment confirm the findings of Duhault et al., (1976), that the oral administration of olive oil produced hyperlipidemic conditions in rats. The increase in triglyceride was more marked as compared to other lipid levels. As shown in Table 4, two hour HMG treatment significantly checked the rise in triglyceride, phospholipid and free fatty acids by 19%, 26% and 20% respectively. HMG treatment for 6 hours was more effective in counteracting hyperlipidemia in rats. The HMG-induced lipid lowering was significant to the extent of 31% in triglyceride, 16% in cholesterol, 25% in phospholipid and 24% in free fatty acid levels. The liver weights in both the sets of experiment were unchanged and the animals remained active during the experimental period.

The Protective Effect of HMG in Alloxan-Induced Diabetes in Rats

Male albino rats weighing 100-150 gm were conditioned on basal diet prior to the start of the experiment. They were made diabetic by a single subcutaneous injection of 180 mg alloxan/kg body weight. The rats were fasted for 48 hour

Table 5. Effect of HMG on Alloxan-Induced Diabetic Rats

	Basal group	Diabetic group	HMG-treated diabetic group	Per cent reduction	p-value
Serum (mg/100 ml)					
Cholesterol	122.7 ± 7.5[a]	137.6 ± 5.7	121.5 ± 2.5	12	$p < 0.02$
Triglycerides	60.1 ± 7.0	168.4 ± 8.1	108.5 ± 11.5	36	$p < 0.001$
Phospholipids	105.4 ± 4.7	116.0 ± 9.9	97.5 ± 5.9	16	$p < 0.05$
Free Fatty Acids	9.3 ± 1.0	25.4 ± 3.7	19.6 ± 1.5	23	$p < 0.02$
Glucose	81.6 ± 4.2	208.5 ± 16.6	163.3 ± 8.4	22	
Liver (mg/gm)					
Cholesterol	3.57 ± 0.41	3.13 ± 0.37	2.75 ± 0.35	11	$p < 0.05$
Triglycerides	8.55 ± 0.86	9.13 ± 1.10	7.56 ± 0.73	16	N.S.
Phospholipids	29.6 ± 0.39	36.7 ± 1.5	34.9 ± 2.3	5	N.S.
Liver weights (gm)	4.0 ± 0.3	3.3 ± 0.25	3.70 ± 0.24	-	-

[a]Mean ± Standard error expressed for five rats.

N.S. = Not Significant

before alloxan administration. The alloxan solution was made just prior to injection and used as quickly as possible. Urine sugar was checked at regular intervals from fifth day. Insulin injection i.p. 0.5 unit/130 gm rat, was adjusted daily for glucosuria $^{++}$ or $^{+++}$ (test tape) and stopped 18 hour before sacrifice. The animals with marked hyperglucosuria were selected for experiment and were divided into 2 groups of 5 animals each. The animals were sacrificed fifteen days after the alloxan injection. From the tenth day onwards the treated animals were given i.p. HMG at the concentration of 50mg/kg/day. The control group received an equal volume of saline. On the last day blood was withdrawn after an overnight fast. Five normal rats were also sacrificed at the same time for comparison. Various lipid paremeters were determined in serum and liver. Glucose content of serum was also determined.

The diabetic state induced in rats with alloxan was characterized by increased food intake, hyperglucosuria, hyperglycemia and by a decrease in body weight gain and liver weight. Diabetic rats had marked increase in cholesterol, triglyceride free fatty acid and phospholipid levels in serum. In liver triglycerides and phospholipids increased whereas cholesterol decreased. As shown in Table 5, HMG effectively protected the diabetogenic action of alloxan by decreasing cholesterol, triglyceride, phospholipid and free fatty acids in serum by 12%, 36%, 16% and 23% respectively. Hepatic cholesterol lowered significantly to the extent of 11%.

An insignificant reduction was observed in hepatic triglyceride and phospholipid levels. The maximum effect of HMG was observed in triglyceride concentrations in serum indicating a beneficial effect of HMG. The mean blood glucose values of diabetic animals receiving HMG were much smaller than those of the untreated diabetic controls. It was interesting to note that the physical condition of diabetic rats improved on HMG treatment. The liver weight decreased in diabetic animals and remained unaltered on treatment with HMG.

Protective Effect of HMG on Carbon tetrachloride - Induced Fatty Liver in Rats

With slight modification the method used was essentially that of Vaishwanar et al.,(1972). Thirty rats weighing 110-140 gm were fed basal diet and water ad libitum. In this study intra muscular injections of carbon tetrachloride 3 ml/kg/day (1:1 v/v) in mineral oil and 50 mg HMG /kg dissolved in normal saline were used. The effect of HMG on carbon tetrachloride treated animals was studied at intervals of 48 and 168 hours. In 48 hour study, the rats received simultaneous injections of carbon tetrachloride i.m. and HMG i.p. 50 mg/kg at an interval of 0,6,24 and 30 hours. The control group received similar doses of carbon tetrachloride and an equal volume of saline at similar intervals. Animals in both the groups were sacrificed at the end of 48 hours. The animals were kept fasting during the experimental period. In 168 hour study, the treated animals received simultaneous injections of carbon tetrachloride and HMG daily for seven days. The control group received

Table 6. Effect of HMG on Carbon tetrachloride-Induced Fatty Liver in Rats: 48 hour study

	Groups			% change	p-value
	Normal	Control	Treated		
Serum (mg/100 ml)					
Cholesterol	69.7 ± 7.0[a]	50.0 ± 2.0	60.0 ± 1.2	20	p = 0.01
Triglycerides	64.4 ± 2.9	42.0 ± 1.0	47.0 ± 2.0	11	N.S.[b]
Phospholipids	116.0 ± 7.5	82.0 ± 4.0	96.2 ± 3.3	17	N.S.
Liver (mg/gm)					
Cholesterol	3.5 ± 0.4	7.6 ± 0.4	7.1 ± 0.3	7	N.S.
Triglycerides	17.8 ± 1.9	63.0 ± 5.0	54.0 ± 7.0	14	N.S.
Phospholipids	25.2 ± 1.8	31.0 ± 1.4	27.0 ± 2.0	13	p = 0.1
Liver weight (gm)	2.8 ± 0.1	4.6 ± 0.3	3.6 ± 0.4	21	p = 0.1

[a]Mean ± Standard Error expressed for six rats

[b]N.S. = Not significant

Table 7. Effect of HMG on Carbon Tetrachloride-Induced Fatty Liver in Rats: 168 hour Study

Details	Groups			% change	p-value
	Normal	Control	Treated		
Serum (mg/100 ml)					
Cholesterol	69.7 ± 7.0[a]	43.0 ± 5.5	61.8 ± 3.2	40	$p < 0.01$
Triglycerides	64.4 ± 2.9	32.7 ± 0.6	42.6 ± 1.2	30	$p < 0.0001$
Phospholipids	116.0 ± 7.5	60.0 ± 5.4	89.0 ± 11.0	46	$p < 0.02$
Liver (mg/gm)					
Cholesterol	3.5 ± 0.4	13.0 ± 0.7	10.9 ± 0.2	16	$p < 0.01$
Triglycerides	17.8 ± 1.9	71.5 ± 6.2	55.4 ± 3.0	23	$p < 0.02$
Phospholipids	25.2 ± 1.8	41.4 ± 1.9	35.8 ± 2.1	14	$p < 0.05$
Liver weights (gm)	2.8 ± 0.1	4.1 ± 0.2	3.1 ± 0.1	25	$p < 0.01$

[a]Mean ± Standard error expressed for six rats.

carbon tetrachloride and saline only. The animals were sacrificed one hour after the last treatment. Six normal rats of similar body weight were also sacrificed for comparison purposes.

In agreement with the earlier reports, cholesterol, triglyceride and phospholipid levels substantially decreased in serum in carbon tetrachloride treated rats. Moreover, these lipid parameters increased in liver in both the experiments (Vaishwanar et al., 1972). Table 6 presents the concentration of various lipid levels in serum and liver for 48 hour study. HMG elevated serum cholesterol significantly to the extent of 20%. The triglyceride and phospholipid levels were, however, insignificantly increased due to HMG treatment. In liver, cholesterol and triglyceride levels were insignificantly lowered whereas the decrease in phospholipid level was significant. In 168 hour study, as evident from Table 7, the protective effect of HMG was even more pronounced. Cholesterol, triglyceride and phospholipid levels in serum were insignificantly increased by 40%, 30% and 46% respectively. Furthermore, HMG significantly prevented the rise in all these lipid parameters in liver. The observed decrease in liver weight due to HMG administration in carbon tetrachloride treated rats was statistically significant. The extent of reduction was 21% in 48 hour study and 25% in 168 hour study. The animals tolerated the treatment relatively well. Their average food consumption and body weight

remained approximately constant in both control and test animals.

Effect of HMG on Cobalt Chloride - Induced Hyperlipidemia in Rats

Twelve male albino rats (110-140 gm) were maintained on basal diet for 2-3 weeks. They were divided into two equal groups. The treated animals were given simultaneous injections of cobalt chloride i.m. 25 mg/kg body weight/day and HMG i.p. 50 mg/kg in 1 ml saline for seven days. The animals receiving cobalt chloride and 1 ml saline served as paired control. For comparison of lipid values, six normal rats of similar body weight were also sacrificed at the same time. At the end of experiment, the animals were fasted overnight before sacrifice. Various lipid levels were analysed in serum and liver.

The data in Table 8 confirm the findings of Morita et al. (1974) and Ohmichi (1977) that cobalt chloride produces hyperlipidemic conditions in animals. Intramuscular administration of cobalt chloride at the concentration of 25 mg/kg/day for seven days resulted in a marked increase in cholesterol, triglycerides, phospholipids and free fatty acids in serum and liver. HHG treatment significantly counteracts the enhanced lipemic response of cobalt chloride in rats. The magnitude of lowering in serum was 20% in cholesterol, 34% in triglycerides, 23% in phospholipids and 25% in free fatty acids. The decrease in hepatic cholesterol, triglyceride and phospholipid levels were 18%, 22% and 14% respectively.

Table 8. Effect of HMG on Cobalt chloride-Induced Hyperlipidemia in Rats

	Groups			% Reduction	p-value
	Normal	Control	HMG-treated		
Serum (mg/100 ml)					
Cholesterol	122.7 ± 7.5[a]	278.6 ± 13.0	222.8 ± 7.5	20	$p < 0.001$
Triglycerides	60.0 ± 7.0	125.0 ± 3.6	82.9 ± 4.2	34	$p < 0.0001$
Phospholipids	105.5 ± 4.7	135.0 ± 2.8	104.4 ± 3.4	23	$p < 0.0001$
Free fatty acids	9.34 ± 0.9	11.6 ± 1.2	8.7 ± 0.8	25	$p < 0.05$
Liver (mg/gm)					
Cholesterol	3.57 ± 0.4	5.57 ± 0.3	4.56 ± 0.3	18	$p < 0.02$
Triglycerides	8.55 ± 0.86	12.9 ± 0.5	10.1 ± 0.4	22	$p < 0.001$
Phospholipids	29.6 ± 0.4	46.0 ± 1.3	39.7 ± 1.4	14	$p < 0.01$
Liver weights (gm)	4.0 ± 0.3	4.4 ± 0.15	4.0 ± 0.19	9	N.S.[b]

[a]Mean ± Standard error expressed for six rats

[b]N.S. = Not significant

The injections of cobalt chloride were tolerated well with no apparent ill effects on the animals. Their eating habits remained normal and no difference was found in weight gain between control and treated groups. However, an insignificant decrease was recorded in liver weight in HMG treated animals.

Age-Related Responsiveness of Rats to Hypolipidemic Action of HMG

The hypolipidemic effect of HMG was studied in normocholesterolemic rats at varying stages of life. Four groups, each of 10 male albino rats were given free access to basal diet and water. The first group contained animals of 2-3; the second 5-6; the third 9-10; and the fourth 15-20 months. They weighed respectively about 30-50 gm; 80-120 gm; 180-220 gm; 280-300 gm. Sub-groups consisting of 5 animals from each group were injected i.p.50 mg HMG/kg/day in normal saline for two weeks. The remaining 5 animals in each group received an equal volume of saline and served as paired controls. At the end of experiment, the animals fasted overnight were anesthetized with ether and blood withdrawn by cardiac puncture as described in Chapter II. Various lipid parameters were analysed in serum and liver.

The data shown in table 9 summarize the effect of HMG on serum and liver lipids in different age groups. The contents of cholesterol, triglyceride and phospholipids varied with age in serum as well as in liver. In group I, the i.p.

Table 9. Age-Related Responsiveness of Rats to Hypolipidemic Action of HMG.

Details	Group I		Group II		Group III		Group IV	
	2-3 months; 30-50 gm		5-6 months; 80-120 gm		9-10 months; 180-220 gm		12-18 months; 280-300 gm	
	control	HMG-treated	control	HMG-treated	control	HMG-treated	control	HMG-treated
Serum (mg/100 ml)								
Cholesterol	48.5±4.1[a]	41.6±5.4[f] (14)	60.2±5.6	50.5±5.9[f] (16)	86.2±4.8	65.0±6.3[d] (25)	105.6±6.0	86.5±9.6[d] (18)
Triglycerides	22.3±1.9	17.8±1.4[e] (20)	39.4±4.2	31.3±3.0[e] (21)	50.6±6.0	36.0±3.4[c] (28)	72±5.4	52.7±3.0[c] (28)
Phospholipids	86.4±8.7	75.7±7.0[f] (12)	129.0±6.6	105.5±8.0[e] (18)	163.0±16.0	133.5±17.0[f] (18)	183.3±14.0	155.5±21.0[f] (15)
Liver (mg/gm)								
Cholesterol	1.2±0.24	1.0±0.12[f] (17)	2.14±0.25	1.74±0.27[f] (19)	2.6±0.23	2.0±0.6[f] (23)	3.9±0.58	3.17±0.56[f] (19)
Triglycerides	2.51±0.16	1.89±0.19[d] (24)	3.28±0.17	2.56±0.18[e]	4.6±0.39	2.98±0.3[c] (35)	6.2±0.37	4.3±0.42[d] (30)
Phospholipids	8.58±0.30	8.7±0.79	11.43±1.0	11.0±0.9	15.9±1.0	15.3±1.7	19.2±1.50	17.1±1.0[f] (11)
Liver weights (gm)	1.84±0.08	1.72±0.15	2.52±0.2	2.26±0.09[f] (10)	4.96±0.35	4.38±0.43[f] (12)	7.26±0.14	7.16±0.16[f] (14)

[a]Mean ± Standard error expressed for five rats

[b]Values in parentheses indicate per cent reduction with respect to control group

[c]Significantly different from control group $p < 0.01$; [d]$p < 0.02$; [e]$p < 0.05$

[f]Not significant

administration of HMG lowered triglycerides significantly to the extent of 20% in serum and 24% in liver. The other lipids of both tissues were insignificantly reduced. In group II, HMG caused a significant fall in triglycerides to the extent of 21% in serum and 22% in liver. A significant reduction of the magnitude of 18% was also observed in serum phospholipids. Serum cholesterol and hepatic cholesterol and phospholipids showed a small effect. The animals of group III recorded a significant decrease of about 25% in cholesterol and 28% in triglycerides in serum. Hepatic triglycerides were also significantly lowered to the extent of 35%. Other lipid levels showed either none or an insignificant response to HMG treatment. In group IV, serum cholesterol and triglycerides significantly declined respectively by 18% and 28%. Among hepatic lipids, only the decrease in triglycerides of about 30% was significant. Serum and liver phospholipids as well as cholesterol in liver were insignificantly lowered. The results show that there was a little or no lowering effect of HMG on phospholipid levels. The maximum decrease occured in triglycerides in both serum and liver in all the groups. The HMG-treated groups did not record any significant decrease in liver weight. No change in body weight gain between control and HMG-treated groups was observed.

Effect of HMG on Normocholesterolemic chickens

To evaluate the hypolipidemic action of HMG in normal birds, two groups each of 5 white chickens weighing about

Table 10. Effect of HMG on Serum Lipids of Normocholesterolemic Chickens

Serum Lipids (mg/100 ml)	Control group	HMG-treated group	Per cent Reduction	p-value
Cholesterol	128.5 ± 12.9	94.4 ± 10.0[a]	27	$p < 0.05$
Triglycerides	138.0 ± 7.9	92.1 ± 8.8	33	$p < 0.001$
Phospholipids	295.0 ± 16.2	266.7 ± 14.7	10	N.S.[b]
Free Fatty Acids	14.1 ± 1.5	12.1 ± 1.4	14	N.S.

[a]Mean ± Standard error expressed in mg/100 ml serum for five chickens

[b]N.S. = Not significant

700-800 gm were caged separately. They were fed basal diet and water ad libitum. While the birds in the HMG-treated group were given by gastric intubation 50 mg HMG/kg/day, those in the control group received 2 ml saline only. The treatment was continued daily for one week. At the end of experiment, the animals were fasted overnight and blood withdrawn from arm vein for analysis. Various lipid parameters were determined in serum.

Data presented in table 10 shows that the oral administration of HMG for one week in chickens significantly lowered cholesterol and triglycerides levels in serum to the extent of 27% and 33% respectively. However, HMG had no significant effects on the levels of phospholipids and free fatty acids. These findings are in agreement with earlier observations in normal rats and rabbits (Yousafzai and Siddigi, 1976; Umar and Siddigi, 1976). The results indicate that HMG has marked hypotriglyceridemic action in chickens also. The body weights of treated birds were identical to the controls.

Effect of HMG on Serum Lipids of Chickens treated with Triton WR-1339

Ten chickens weighing about 700-800 gm were divided into two equal groups. They were conditioned on basal diet for two weeks and were fasted for 24 hr prior to the start of the experiment. At the end of 24 hr, all the animals received i.v. Triton WR-1339 at a dose of 300 mg/kg body weight dissolved in 0.15 M NaCl. The experimental animals were given

Table 11. Triton-Induced Hyperlipidemia in Chickens

Serum Lipids	Normal	20 hour		43 hour	
		control	HMG-treated	control	HMG-treated
Cholesterol	128.5 ± 12.9	340.0 ± 9.7	210.6 ± 10.9[a]	318.7 ± 30.0	204.6 ± 14.6
% Reduction			38%		35%
p-Value			$p < 0.0001$		$p < 0.001$
Triglycerides	138.0 ± 7.9	336.0 ± 25.0	229.6 ± 10.6	318.7 ± 21.8	209.8 ± 17.0
% Reduction			32%		26%
p-Value			$p < 0.001$		$p < 0.001$
Phospholipids	295.0 ± 16.2	647.8 ± 39.0	516.0 ± 28.5	520.0 ± 22.0	289.5 ± 29.7
% Reduction			20%		25%
p-Value			$p < 0.01$		$p < 0.001$
Free Fatty Acids	14.1 ± 1.5	17.7 ± 1.9	15.6 ± 1.2	16.56 ± 1.49	14.4 ± 1.5
% Reduction			12%		13%
p-Value			N.S.[b]		N.S.

[a]Mean ± Standard error expressed in mg/100 ml serum for five chickens

[b]N.S. = Not significant

in addition HMG i.p. at the concentration of 50 mg/kg. Each animal of the treated group received two doses of HMG, first immediately after Triton injection and the second 20 hr later. Animals receiving an equal volume of saline i.p. served as controls. Blood was withdrawn 20 hr after Triton administration. Subsequently the animals received second dose of HMG. Blood was withdrawn again 43 hr post-Triton period. Fasting was continued till the end of the experiment. Various lipid contents were analysed in serum.

The data in Table 11 is similar to the findings of Schurr (1972) that the administration of Triton produces hyperlipidemic conditions. The lipid levels of HMG-administered birds were significantly lower in both durations when compared with controls. Cholesterol, triglyceride and phospholipid respectively decreased by 38%, 32% and 20% in 20 hr and 35%, 26% and 25% in 43 hr. Also, an insignificant effect was observed in free fatty acids in both sets of experiment. However, there was no major difference in the extent of lowering in these lipid parameters at 20 hr and 43 hr.

C H A P T E R IV

D I S C U S S I O N

DISCUSSION

In recent years advances in the study of normal and abnormal lipid metabolism have resulted in a broader understanding of the mechanism and effect of hypolipidemic drugs (Fredrickson and Levy, 1972). A clear concept of how lipid is transported in blood has helped to clarify the etiology of lipid disorders, which in turn has provided fresh insights into reasons for the efficacy of lipid lowering agents. Thus, the management of hyperlipidemia has become increasingly precise and the selection of therapy more rational. The relationship between elevated serum lipid levels and the risk of developing CHD is well established. Because of the correlation of heart disease with elevated serum cholesterol levels, most attempts at dietary intervention have centered upon limitation of dietary fat in general and saturated fat and cholesterol in particular. The hyperlipidemia in relation to atherosclerosis is associated with increase in lipoprotein concentration (Jones, 1973). Hyperlipidemia and hyperlipoproteinemia could be either due to increased release of VLDL from liver into plasma or the mechanism of its removal from plasma becomes defective. It is known that increased fatty acid and cholesterol biosynthesis can lead to elevate plasma lipoproteins in man and animals. Genetically obese animals that have high lipogenic capacity are also hyperlipidemic

(Maragoudakis et al., 1972). HMG has been shown to act as an inhibitor of cholesterol biosynthesis between HMG-CoA and mevalonate (Rabinowitz and Gurin, 1954) and competitively inhibits the HMG-CoA reductase (Fimognari and Rodwell, 1965). Beg and Lupien (1972) have shown that it inhibits cholesterol biosynthesis. Either oral feeding or more effectively i.p. administration of HMG has been shown to exhibit hypocholesterolemic and hypolipidemic properties in rats (Beg and Siddigi, 196[illegible] 1968) and rabbits (Lupien et al., 1973a; Yusufi and Siddiqi, 1974). HMG has been shown to be effective in the treatment of 3 patients suffering from Type II hyperlipoproteinemia (Lupien et al., 1973b). It has also been implicated in the physiological control mechanism for cholesterol biosynthesis (Saleemuddin and Siddiqi, 1973). Oral administration of 1 gm HMG before the ingestion of whiskey and a fatty meal markedly reduce the elevation of serum triglycerides, β-lipoproteins, phospholipids and cholesterol in man. In rats receiving ethanol and corn oil mixture, HMG also inhibited the increase in post-prandial serum and liver lipids (Yousufzai et al., 1976). In rats, HMG effectively counteracts the lipemic and atherosclerotic response of massive doses of vitamin D_2. It regressed the formation of atheromatous arterial lesions. Furthermore, the significant decrease in serum β-lipoprotein levels on HMG treatment could be due to decrease in VLDL triglyceride and cholesterol levels (Yousufzai and Siddiqi, 1976a). HMG significantly decreased cholesterol,

triglyceride and phospholipid levels in whole serum, serum β-lipoproteins and liver of Triton-induced hyperlipidemic rats. They also observed that 50 mg HMG/kg was equivalent to 200 mg nicotinic acid/kg in offering almost total protection against lipemic response of either Triton or alcohol in rats. It was suggested that HMG exerted its hypolipidemic effect through the inhibition of lipoprotein synthesis (Yousufzai and Siddiqi, 1976a, 1976b). HMG showed a significant cholesterol and triglyceride - lowering effect in the whole serum, serum β-lipoproteins and liver of rats on all type of dietary carbohydrates. The effect was more marked in glucose, fructose, sucrose and lactose. The HMG-induced lowering of lipid parameters in serum β-lipoproteins and also in liver could be either due to inhibition of VLDL synthesis or to VLDL triglycerides release in liver (Yousufzai and Siddiqi, 1977a). Simultaneous administration of HMG for 4 weeks to rats fed 20% saturated fats prevented the rise of serum cholesterol, triglycerides and phospholipids, and cholesterol and phospholipids in liver, aorta and heart. HMG significantly lowered the phospholipids in epididymal fat and brain and triglyceride levels in serum, liver and aorta. The maximum hypolipidemic effect was observed in serum (Yousufzai and Siddiqi, 1977b).

Although a large number of compounds have been found to lower serum cholesterol and triglycerides in man, the drugs used at present are few:clofibrate, nicotinic acid and

cholestyramine. Many patients treated for hyperlipoproteinemia still have elevated serum lipid values on diet and during treatment with the recommended doses of the drugs.

From the preceding paragraph, it is clear that HMG is quite efficient in normalizing tissue lipid levels. The purpose of our study was to throw more light on the hypolipidemic action of HMG in order to understand its possible mode of hypolipidemic action. To obtain sufficient lipid lowering response, a combination with nicotinic acid was also studied. As evident from Table 2, the administration of 10 mg HMG and 40 mg nicotinic acid/kg significantly reduced serum triglyceride levels. Except serum triglycerides, the decrease in all the lipid parameters of serum, liver and aorta was insignificant, whereas the administration of 20 mg HMG or 80 mg nicotinic acid/kg resulted in a significant decrease in cholesterol and triglyceride concentrations in all these tissues. The combined lower dose of the two compounds significantly decreased all lipid parameters in all these tissues. The reduction was remarkedly significant on the administration of combined higher doses of the two compounds. These findings suggest that the reduction in tissue cholesterol and triglyceride concentrations by HMG were dose-dependent. We confirm the earlier findings of Berge et al. (1961); Oro (1970) and Olsson et al. (1975) that the hypolipidemic effect of nicotinic acid is dose-dependent. We find that during the combined treatment, the reduction in all lipid

parameters in serum, liver and aorta was more marked than with either compound alone in these doses. The effect in cholesterol level was more efficient than with either compound alone. Similar results were obtained for triglycerides in these tissues. The effect observed was, therefore, synergistic. On administration of 10 or 20 mg HMG/kg in rats, a significant decrease in average wet weight of liver was observed (Table 3). There was no change in liver weight by the administration of either dose of nicotinic acid. On administration of combined lower or higher doses of compounds, a significant reduction of the same magnitude as with 10 or 20 mg HMG/kg was observed. It is concluded, therefore, that the decrease of liver weight in the combined doses in only due to HMG administration and not due to nicotinic acid. We confirm the earlier findings of Lupien et al. (1973a); Yusufi and Siddiqi (1974); Yousufzai and Siddiqi (1977a, 1977b) that HMG lowers the average liver weight in rats and rabbits than their respective control groups. It is known that in the coronary diseased group, both cholesterol and phospholipids are increased in serum and cholesterol:phospholipid (C/P) ratio was likewise increased. Since the C/P ratio is generally believed to be an index of atherogenecity, the rise in C/P ratio in serum is a prerequisite for atherogenecity (Gresham et al., 1965). A compound capable of lowering C/P ratio will, therefore, help not only in normalizing tissue lipid levels but also atherosclerotic conditions. In our studies, the average C/P ratio, in lower single, higher

single or the respective combined doses of the two compounds decreased in serum, liver and aorta, except liver and aorta of animals treated with 10 mg HMG/kg. The dose-response effect of HMG was also observed in C/P ratio in serum and aorta. The same was true for nicotinic acid in serum. The cholesterol:triglyceride ratio (C/T) increased in serum and liver of animals treated with any dose of HMG or nicotinic acid. The observed HMG-induced decrease in lowering of various lipid parameters and C/P ratio as well as an increase in C/T ratio is in agreement with the earlier findings of Beg and Siddiqi (1968) and Yousufzai (1976). The hypolipidemic response of HMG even for liver and aorta lipids is, therefore, established.

For a number of years, investigations on the mechanism of fat absorption by the intestine have been primarily concerned with the analysis of the contents of the digestive tract and thoracic duct lymph following a fat meal. Transport of exogenous triglycerides in chylomicrons is highly efficient so that chylomicronia ensues within few hours after each fatty meal. This process can account for transport of as much as several hundred grams of triglycerides daily. The pathway of transport of triglycerides in blood plasma and its regulation provides a reasonably firm basis for evaluating the action of drugs that reduce triglyceride levels.

It has been suggested that fructose in drinking water increases the intestinal absorption of lipids especially

triglycerides. Similar findings have been observed in this laboratory (Yousufzai, 1976). They have found that HMG reduces cholesterol, triglyceride and phospholipid levels in whole serum, serum β-lipoproteins and liver of rats receiving 10% fructose in drinking water. In accord with Duhault et al. (1976) the intestinal absorption of olive oil increases serum lipids especially triglycerides. The data given in Table 4 confirm the findings of Duhault et al., that the oral administration of olive oil produces hyperlipidemic condition in rats. At 2 and 4 hr after administration of olive oil, there was no major difference in rise of lipid levels in serum. Oral administration of HMG significantly reduced the rise in these lipids. HMG treatment for 6 hr was more effective in counteracting olive oil-induced hyperlipidemia in rats. Among several mechanisms which could explain the increase in intestinal absorption of fat and thereby produce hypertriglyceridemic conditions in rats on oral administration of olive oil, the influx of exogenous fatty acids of olive oil could be an important factor in stimulating the VLDL synthesis in the intestine as well as in liver (Nikkila, 1969). Since HMG is capable of counteracting the olive oil-induced hypertriglyceridemic effect only it would be reasonable to conclude that like nicotinic acid (Levy et al., 1972) HMG could decrease VLDL production. This would also explain the observed decrease in body weight of rats treated with HMG and given high fat (Yousufzai and Siddiqi, 1977b) or high

carbohydrate diets (Yousufzai and Siddiqi, 1977a), which was interpreted in terms of HMG to counteract high fat or high carbohydrate-induced obesity. The possibility of its inhibitory effect on pancreatic lipase (Bernier, 1975) and phosphatides phosphohydrolase (Bowley and Brindly, 1976), however, can not be ruled out.

There is now sufficient evidence that the diabetic condition increases the risk of atherosclerosis in coronary, cerebral and peripheral arteries (Carlson and Bottiger, 1972; Garcia et al., 1974). Experimental diabetes induced in laboratory animals by alloxan or streptozotocin is accompanied by marked changes in lipid metabolism, notably an elevation in serum triglycerides (Meier et al., 1972; Reaven and Reaven, 1974) and a decrease in hepatic cholesterol synthesis (Cayen et al., 1975). These abnormalities are associated with atherosclerosis in diabetes (Santen, 1972). It has been reported that hypertriglyceridemia of the diabetic rat is caused by an accelerated removal mechanism of VLDL triglycerides (Whiting et al., 1977). Other studies indicate, however, that increased VLDL synthesis is the most important cause of hypertriglyceridemia in human diabetes (Nikkila and Kekki, 1973). The decrease in cholesterol synthesis was ascribed to the suppression of HMG-CoA reductase (White, 1970). In view of these changes we considered it of interest to use the alloxan-induced diabetic rats to investigate further the mode(s) of action of HMG. The diabetic state induced in

rats was characterized by hyperglucosuria, hyperglycemia, increased food intake and by a decrease in body weight gain and liver weight. As shown in Table 5, diabetic rats had increase in serum cholesterol and this was associated with a comparable increase in phospholipid levels. Hepatic cholesterol showed a small decrease. This could be due to the inhibition of cholesterol biosynthesis in diabetes. The increase in serum cholesterol coupled with the decrease in hepatic cholesterol synthesis found in diabetic rats can be explained by redistribution of cholesterol from liver to blood and by impaired catabolism and removal of cholesterol from the body (Sadahiro et al., 1970). Administration of HMG caused a significant decrease in serum cholesterol levels in diabetic rats whose rate of hepatic cholesterol synthesis was already suppressed (White, 1970). A significant decrease in cholesterol was observed in liver. This suggests that treatment with HMG suppressed the remaining cholesterogenic activity via inhibition of HMG-CoA reductase (Beg and Lupien, 1972). The hypertriglyceridemia observed in diabetic rats in the present study is in accordance with previous reports (Meier et al., 1972; Reaven and Reaven, 1974). Liver triglyceride and phospholipid levels were virtually unaltered. Intraperitoneal administration of HMG lowered the elevated serum triglycerides; the extent of this antihypertriglyceridemic effect was comparable to the effect produced by HMG in normal rats (Beg and Siddiqi, 1968). The opposite effects of diabetes and HMG on serum

triglycerides in rats may be due to the opposite effects on α-glycerophosphate dehydrogenase. This suggestion is based on the reports that experimental diabetes increases hepatic triglyceride synthesis by decreasing the activity of α-glycerophosphate dehydrogenase (Corder and Kalkhoff, 1969). Furthermore, since the activity of lipoprotein lipase and/or adipolytic lipase is decreased in diabetic rats at the site of triglyceride hydrolysis (Pykalisto et al., 1974; Van Tol, 1977), the opposing effects of HMG on these enzymes in serum could provide an additional contribution to the antihypertriglyceridemic activity of HMG in diabetic rats. The circulating free fatty acid levels increased in diabetic rats. This is in agreement with the earlier findings that the free fatty acid release from depot fat is increased in diabetes (Cayen et al., 1975). The free fatty acid levels were decreased by treatment with HMG. This finding suggests that the hypolipidemic activity of HMG could be due to an inhibition of free fatty acid release from adipose tissue. The mean glucose values of diabetic animals receiving HMG were also much smaller than those of untreated diabetic controls. It was interesting to note that the physical condition of diabetic rats improved on HMG treatment. This suggests that the administration of HMG improved glucose tolerance without associated weight loss (Jaillard and Riveline, 1975).

One experimental approach in the study of regulation of lipoprotein metabolism is through the exploration of the

mechanism of fatty liver production. An inverse relationship between hepatic triglyceride accumulation and serum lipoprotein concentration has been demonstrated in rats treated with carbon tetrachloride (Recknagel, 1967). In the present study it was observed that animals treated with carbon tetrachloride showed tremendous rise in hepatic lipids especially triglycerides. There was a simultaneous decrease in these lipids in serum. This could be due to a defective enhancement in the synthesis and/or availability of lipid in liver for lipoprotein synthesis. A defect in conjugation of protein with lipid moities or a blockage in the secretary mechanism of lipoproteins in the endoplasmic reticulum of liver is also possible (Lombardi, 1965; Vaishwanar, 1976).

Essentially all the drugs under investigation as hypolipidemic agents prevent orotic acid fatty liver. Although not yet unequivocally established, it has been suggested that such a hypolipidemic effect could arise either by counteracting hepatic inhibition of VLDL synthesis or fascilitating the release of VLDL from liver to plasma (Elwood et al., 1972). In a previous study from this laboratory, it was reported that like nicotinic acid, cholestyramine and pyrazonic acid, HMG failed to prevent orotic acid-induced fatty liver in rats (Yousufzai and Siddiqi, 1977c). This observation led to conclude that the hypolipidemic activity of HMG is mediated through the inhibition of synthesis of β-lipoproteins. To confirm these results

the effect of HMG on carbon tetrachloride - induced fatty liver was studied. Like nicotinic acid (Vaishwanar et al., 1972), the administration of HMG to rats reverse the changes in different lipid fraction in serum and liver caused by carbon tetrachloride. In 48 hr study, the observed per cent change was significant only in serum cholesterol and hepatic phospholipids (table 6). In 168 hr study, as evident from table 7, HMG significantly decreased cholesterol, triglyceride and phospholipids in liver. On the other hand, these lipid levels were increased in serum. The significant change in these lipid levels show that somehow it interferes in β-lipoprotein synthesis. It could be postulated that HMG was improving the defective synthesis or availability of lipids or conjugation mechanism for lipoprotein synthesis. It is possible that HMG was fascilitating the secretion of lipoproteins from liver to plasma. The observed decrease in liver weight due to HMG in carbon tetrachloride treated rats was significant in both studies. Unlike orotic acid-induced fatty liver, it indicates a beneficial effect of HMG in counteracting the fatty liver conditions in rats. No change in body weight between HMG-treated and control group, and a change in lipid levels in serum and liver suggests that HMG probably inhibited the depot fat mobilization, thereby decreasing the availability of triglycerides and free fatty acids for the synthesis of different lipid fractions in liver. Since carbon tetrachloride is known to impair the secretory

mechanism of hepatic parenchymal cells (Vaishwanar, 1972) and HMG counteracts the fatty liver caused by carbon tetrachloride, it appears that HMG checks the synthesis of β-lipoproteins rather than the release of VLDL. However, the effect of HMG on release of β-lipoproteins can not be ruled out.

According to Eaton and Kipnis (1969a, 1969b) cobalt ion produces an endogenous hyperlipidemia which is virtually indistinguishable from 'carbohydrate-induced lipemia' and is independent of dietary considerations. Later on in 1972, Eaton reported that endogenous hyperlipidemia of both carbohydrate feeding and cobalt chloride treatment is initiated by insulin-mediated stimulation of hepatic protein synthesis followed by release of lipid carrying protein into circulation. The serum of rabbits was characterized as grossly lipemic with the predominant abnormality being an increase in VLDL transport protein and its triglyceride component.

Our date in table 8 confirm the findings of Eaton and others (Lempert and Levina, 1974; Ohmichi, 1977) that cobalt chloride produces hyperlipidemic conditions. In this study intramuscular administration of cobalt chloride at the concentration of 25 mg/kg/day for seven days resulted in a marked increase in cholesterol, triglycirides, phospholipids and free fatty acids in serum and liver. Treatment of rats with 50 mg HMG/kg/day effectively counteracted the enhanced lipemic response of cobalt chloride. All these lipids

decreased significantly in serum as well as in liver. HMG has been found to prevent carbohydrate-induced lipemia (Yousufzai and Siddiqi, 1977a). This could either be due to inhibition of VLDL synthesis or to VLDL triglyceride release in liver. It has been shown that HMG was unable to overcome orotic acid-induced fatty liver changes in rats (Yousufzai and Siddiqi, 1977c). This may rule out the possibility of HMG inhibiting the release of VLDL and LDL. Since lipoproteins participate in mobilization of liver lipids (Levy et al., 1972; Tzagournis, 1978) and HMG significantly lowers lipids in animals receiving cobalt chloride, it appears that HMG checks VLDL synthesis rather than the release of VLDL. This is also evident from the observation that the decrease in serum lipids was not accompanied by rise in liver lipids as reported earlier (Yousufzai and Siddiqi, 1977b).

Aging in man is associated with a variety of changes in lipid metabolism, some of which have been closely linked to the development of atherosclerosis. Both intrinsic aging processes and the environmental factors (specially diet) operative over many years apparently act in concert with unknown genetic factors to produce the very prevalent multi-factorial disease atherosclerosis (Siddiqi and Yousufzai, 1977). Carlson in 1973 studied the distribution of plasma triglycerides and cholesterol in the lipoprotein fractions of males aged 30-55 years with myocardial infraction or perepheral atherosclerosis. They found that there was an

elevation of triglycerides in the VLDL fraction and in LDL fraction with increasing age. Cholesterol was significantly elevated in the VLDL and to a lesser extent in the LDL. There was a consistent decrease in HDL fraction. Subjects over 55 years of age showed a similar pattern, here considerably greater in the myocardial infarction subjects than in those with perepheral atherosclerosis.

The work presented here was performed to demonstrate age-related alterations in the responsiveness of mature rats to HMG and to emphasize the importance of age as a factor in assessing the compound response. Effectiveness of HMG were evaluated in 2-3; 5-6; 9-10; and 15-20 months old, male rats. As shown in table 9 the concentrations of cholesterol, triglyceride and phospholipid increased with age in serum and liver. Administration of HMG to 2-3 month old rats caused a significant decrease in serum and liver triglycerides only. Cholesterol and phospholipids in serum and liver showed no or a small effect. In 5-6 month old rats, HMG administration significantly decreased serum and liver triglycerides and serum phospholipids. The 9-10 month old rats responded slightly greater. They showed significant effect of HMG in serum and liver triglycerides. In this group, serum cholesterol was also significantly lowered. Similar results were obtained in 15-20 month old rats i.e., cholesterol and triglycerides in serum declined significantly and among hepatic lipids, the decrease in triglycerides was significant.

It has already been established that HMG inhibits cholesterol synthesis (Fimognari, 1964; Beg and Lupien, 1972) and may interfere at some stage of fatty acid synthesis like CPIB and TPIA (Maragoudakis, 1969). The decrease in serum and liver triglycerides caused by HMG administration in all groups suggests that like nicotinic acid (Barboriak and Meade, 1971) and CPIB (Fallon et al., 1972) HMG may inhibit the mobilization of free fatty acids from endogenous lipid stores. This could reduce the uptake of free fatty acid in liver resulting in decreased hepatic formation of VLDL.

The older animals used in these studies were in no sense "old", yet insignificant differences in responsiveness between the age groups of approximately 3 and 9 months old rats were evident. It is quite likely that 2 week duration of HMG treatment was not enough to induce maximal differences among various age groups. Several mechanisms are probably involved in this phenomenon including alterations in the compound distribution, metabolism, elimination and target organ sensitivity. Thus, in pharmacological studies, the age of the animals employed should be considered as one of the experimental variables to be controlled. No change in weight gain between control and HMG-treated animals suggest that HMG does not retard the growth of young rats.

Hypolipidemic properties of HMG have been shown in rats (Beg and Siddiqi, 1967; 1968; Yousufzai and Siddiqi, 1976a, 1977b), rabbits (Lupien et al., 1973a; Yusufi and

Siddiqi, 1974) and man (Lupien, 1973b; Yousufzai and Siddiqi, 1976a,b). The experiment in rats and rabbits indicated that HMG decreased lipid levels in serum, liver and aorta and prevented the atheromatous plaque formation. Since no information was available about the hypolipidemic effect of HMG in birds, we report here that HMG has lipid lowering effect in normocholesterolemic chickens also. The results in table 10 show that the oral administration of HMG at the concentration of 50 mg/kg for 1 week to normocholesterolemic chickens significantly lowered cholesterol and triglyceride levels in serum. The free fatty acids and phospholipids were insignificantly decreased, which might be due to the short duration of HMG treatment. It has been known that HMG inhibits the biosynthesis of cholesterol (Beg and Lupien, 1972). Since all the fractions were affected, the action seems likely on lipoproteins as suggested by earlier workers in this laboratory (Yousufzai and Siddiqi, 1977a). Moreover, reduction was most marked in triglycerides, suggesting an effect on VLDL and LDL. Because HMG exerts profound hypolipidemic effect in chickens, the lipid-lowering action does not appear to be species-specific.

Several cyclic analogues of clofibrate have been reported by Witiak et al., 1975 and Goldberg et al., 1977, to possess hypotriglyceridemic and/or hypocholesterolemic properties in the Triton WR-1339-induced hyperlipidemic rat model. Schurr (1972) have described a screening procedure

for hypolipidemic compounds in Triton WR-1339-induced hyperlipidemic rats. Garattini et al., (1961) and Paoletti (1962) suggested the use of Triton-induced hyperlipidemia as an approach to screen or to differentiate the mechanism of action of hypolipidemic drugs. Their tests were of two kinds. In the 'first phase' test the drug was given i.p. at the same time as Triton, and a decrease in hyperlipidemia 8 hr later compared to controls was taken as evidence of activity resulting from an inhibition of increased synthesis of cholesterol and fatty acids. In the 'second phase' test the drug was given 22-24 hr after Triton, and the decrease in blood lipids 8 hr later compared to controls was interpreted as an indication of the drugs ability to accelerate lipid removal. Yousufzai and Siddiqi (1976a) used Triton-induced hyperlipidemia in rats to elucidate the mechanism of action of HMG. The results of our experiment in chickens are shown in table 11. Administration of Triton produced Hyperlipidemic condition in chickens. HMG at both durations (20 hr and 43 hr post-Triton) significantly decrease the rise in cholesterol, triglyceride and phospholipids in serum. However, an insignificant effect was observed in free fatty acids. It was of interest that there was not much difference in the per cent decrease of various lipid levels 20 hr and 43 hr after Triton administration. This suggests that HMG effectively counteracts the hyperlipidemic response only when given with Triton. This conclusion is in accordance with the earlier findings

of Yousufzai and Siddiqi (1976a) where the animals were rats. Among several possibilities of mechanism of action of HMG reported earlier, it has been emphasized that hypolipidemic response of HMG could involve a shift in lipoprotein spectrum (Lupien et al., 1973a; Yusufi and Siddiqi, 1974). Recently it has been shown that HMG has no effect on orotic acid fatty liver in rats (Yousufzai and Siddiqi, 1977c). Since Triton is known to physically alter VLDL, and HMG is capable of counteracting Triton-induced hyperlipidemia, the possibility of HMG exerting its hypolipidemic effect through the inhibition of lipoprotein synthesis appears more plausible. Furthermore, the method used detects compounds which inhibit lipid biosynthesis or its catabolism (Schurr et al., 1972), the hypolipidemic activity of HMG may be mediated through its effect on lipid metabolism. Fogelman et al., 1975 have recently shown that normally 12% of mevalonic acid is catabolized through a shunt pathway in mammalian system involving trans-3-methyl-glutaconyl CoA. A derailment in the operation of this pathway might explain the hypercholesterolemic condition in animals and man. In view of earlier reports on hypolipidemic activity of HMG (Lupien, 1973a; Yousufzai and Siddiqi, 1977c), it is tempting to suggest, therefore, that HMG may in some way correct the derailed pathway.

Our studies strengthen the belief that HMG has an antihyperlipidemic effect in animals and man. However, much work is to be done to arrive at the exact mechanism(s) through

which HMG exerts its hypolipidemic and hypolipoproteinemic properties. It is tolerable, non-toxic and effective in experiments presented in this thesis. This is not surprizing as HMG is a natural metabolite arising *in vivo* by deacylation of HMG-CoA (Dekker *et al.*, 1958). With these considerations the potentiality of HMG as a future drug for CHD is great. Further clinical studies are desirable to establish its therapeutic value. The multiple action of HMG can be described in the following ways.

Details	Possible Mode of Action
1. Hypercholesterolemic rats	Inhibition of cholesterogenesis and lipogenesis.
2. Olive oil-induced hypertriglyceridemic rats	Inhibition of intestinal absorption of fat Inhibition of increased triglyceride synthesis Inhibition of the activity of pancreatic lipase Inhibition of VLDL production
3. Carbon tetrachloride-induced fatty liver in rats	Like nicotinic acid, it inhibits the lipoprotein synthesis Inhibition of the mobilization of depot fat
4. Cobalt chloride-induced hyperlipidemic rats	Inhibition of VLDL synthesis
5. Alloxan-induced diabetic rats	Inhibition of cholesterogenesis Increase in the activity of α-glycerophosphate dehydrogenase Increase in the activity of lipoprotein lipase Inhibition of increased free fatty acid release in diabetes

Details	Possible Mode of Action
6. Normocholesterolemic rats and chickens	Inhibition of cholesterogenesis and lipogenesis
7. Triton-Induced hyperlipidemia in chickens	Inhibition of increased synthesis of cholesterol and fatty acids Inhibition of synthesis of lipoproteins Correction in derailed mevalonic acid shunt pathway in mammalian system.

C H A P T E R V

R E F E R E N C E S

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ABBREVIATIONS

CHD : coronary heart disease

CPIB: ethyl p-chlorophenoxyisobutyrate

HDL : high density lipoproteins

HLP : hyperlipoproteinemia

HMG : 3-hydroxy-3-methylglutaric acid

i.m.: intramuscular

i.p.: intraperioteneal

i.v.: intravenous

LDL : low density lipoproteins

s.c.: subcutaneous

TPIA: tetralylphenoxyisobutyrate

VLDL: very low density lipoproteins